ROLE OF AUTOPHAGY IN ALCOHOL-INDUCED ADIPOSE ATROPHY AND LIVER INJURY

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ABSTRACT

Alcoholic liver disease (ALD) is a worldwide health issue claiming 2 million lives per year. The pathogenesis of ALD is characterized by steatosis, alcoholic hepatitis, fibrosis, cirrhosis, and eventually hepatocellular carcinoma. There is no ideal treatment other than liver transplantation for late stage of ALD, and novel treatment targets especially for early stage of ALD are still needed. In recent years, increasing evidence implicates the role of adipose-liver axis in in the pathogenesis of ALD. Chronic alcohol consumption leads to increased lipolysis, adipose tissue atrophy, and proinflammatory adipokine secretion. However, the mechanisms of how adipose tissue is affected by alcohol and contributes to ALD are largely unknown.

In this study, I found that chronic-plus-binge alcohol treatment, a model resulting in liver injury and steatosis, led to smaller adipocytes and decreased adipose tissue mass. Mechanistically, I found that chronic-plus-binge alcohol inhibited mammalian target of rapamycin (mTOR)/Akt signaling pathways and enhanced autophagy degradation in epididymal adipose tissue. Although the adipose-specific autophagy-related gene 5 knockout (A-Atg5 KO) mice with adipose autophagy deficiency did not present adipose atrophy at basal condition, they were resistant to alcohol-induced adipose tissue atrophy. Moreover, A-Atg5 KO mice had increased adipocytes with multilocular lipid droplets in subcutaneous white adipose tissue. Interestingly, A-Atg5 KO mice were more resistant to alcohol-induced liver injury, though they still developed alcohol-induced liver steatosis. Alcohol metabolism and reactive oxygen species generation in liver was not affected in A-Atg5 KO mice. While the chronic-plus-binge alcohol increased liver mRNA levels of several proinflammatory genes in both wild-type and A-Atg5 KO mice, A-Atg5 KO mice had significantly decreased induction of *Ccl2*. Compared with wild-type mice, A-Atg5 KO mice had similar serum lipids (triglycerides, free fatty acids, free glycerol), but had higher basal levels

of adiponectin and fibroblast growth factor 21 (FGF21). Additionally, chronic-plus-binge alcohol did not induce inflammation or cell death in white adipose tissues.

I next used cultured 3T3-L1 preadipocytes to further determine the role of autophagy in adipocyte differentiation and how adipogenesis is affected by alcohol. I found that there was increased autophagy degradation along with increased levels of mitochondria proteins during preadipocyte adipogenesis. Using fluorescence microscopy, I found that elongated, sharp mitochondria signal became diffused and enhanced in mature adipocytes. Electron microscopy analysis revealed that the number of elongated mitochondria with clear cristae structure largely disappeared while electron-dense vacuoles containing undegraded cellular components frequently appeared during adipogenesis. Long-term cotreatment using chloroquine, a lysosome inhibitor that blocks autophagy degradation, sufficiently blocked 3T3-L1 adipogenesis. Intriguingly, in chloroquine-treated cells typical mitochondria still decreased while the electron-dense vacuoles containing undegraded cellular components further increased. Moreover, long-term ethanol or acetaldehyde treatment did not inhibit morphological changes or lipid droplet accumulation during adipogenesis in 3T3-L1 cells. Interestingly, short-term ethanol but not acetaldehyde treatment induced autophagic flux, and long-term ethanol but not acetaldehyde treatment increased mitochondria protein levels. The change in mitochondria morphology during adipogenesis was not affected by long-term ethanol or acetaldehyde treatment. These data suggest that autophagy is required for the proper adipogenesis of cultured 3T3-L1 cells, which is associated with dynamic mitochondrial remodeling.

In summary, I characterized the effect of chronic-plus-binge alcohol treatment on adipose morphology, mTOR signaling and autophagy in mice. I demonstrated that chronic-plus-binge alcohol inhibited mTOR and increased autophagic flux and adipose tissue atrophy in mice. I further demonstrated that mice with chronic adipose tissue autophagy deficiency were more

resistant to alcohol-induced adipose atrophy. These data indicate that autophagy activation contributes to the adipose dysfunction induced by alcohol. In addition, I also demonstrated that A-Atg5 KO mice were more resistant to alcohol-induced liver injury likely due to the increased secretion of adiponectin and FGF21 at the basal levels of A-Atg5 KO mice. These data thus support an important role of adipose-liver axis in the pathogenesis of ALD. These studies further support the notion that targeting adipose tissue autophagy may be helpful in improving alcohol-induced liver injury.

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CHAPTER 1. INTRODUCTION

This chapter has been previously published as two separate articles and is reprinted here with adaptations and new material with permission. The original articles were cited as:

Li, Y., S. Wang, H. M. Ni, H. Huang and W. X. Ding (2014). "Autophagy in Alcohol-Induced Multiogran Injury: Mechanisms and Potential Therapeutic Targets." Biomed Res Int **2014**: 498491.

Li, Y. and W. X. Ding (2017). "Adipose Tissue Autophagy and Homeostasis in Alcohol-Induced Liver Injury." Liver Res **1**(1): 54-62.

1.1 Adipocytes and Adipose Tissues

In mammals, adipose tissues or fat tissues are mainly categorized into white adipose tissue (WAT) and brown adipose tissue (BAT) which have distinct origin, morphology and functions. Adipocytes, also known as lipocytes or fat cells, are the predominant cells composing adipose tissues. Adipocytes are found not only in stereotypical fat depots but also in other locations like loose connective tissues. Here we mainly discuss adipocytes in adipose tissue.

1.1.1 White Fat

White adipocytes mainly originate from myogenic factor 5 (Myf5)-negative progenitors (Seale et al. 2008) and in adulthood from endothelial cell lines (Tran et al. 2012). Later evidence suggests that a small subset of white adipocytes originate from Myf5-positive progenitors (Sanchez-Gurmaches et al. 2012, Shan et al. 2013). White adipocytes are characterized by a single large lipid droplet (LD), and a nucleus and scarce cytoplasm squeezed into the rim of cells (Fig.1.1.1A).

The main function of WAT is to store energy in the form of LD. When there is sufficient nutrient intake, the insulin receptor on WAT is activated by insulin released from pancreas, which leads to a dephosphorylation cascade and inactivates hormone-sensitive lipase (HSL), favoring storage of triglycerides (TGs) in LDs. When the body needs energy, the stored TGs break down to fatty acids (FAs), which are taken up by muscle and cardiac tissue as a fuel source. The breakdown of TGs also generates glycerol, which is taken up by the liver for gluconeogenesis. WAT also has other important roles such as a thermal insulator helping to maintain body temperature (Alexander et al. 2015); a hormone receptor in response to insulin (Bluher et al. 2002), growth hormone (Berryman et al. 2011), norepinephrine (White and Engel 1958), and glucocorticoids (MacDougald et al. 1994); and an endocrine organ secreting adipokines including

adiponectin (Maeda et al. 1996), leptin (Zhang et al. 1994), resistin (Steppan et al. 2001), and apelin (Boucher et al. 2005). Obesity and overweight are usually accompanied with increased size and number of white fat cells. WAT is further categorized as subcutaneous WAT (sWAT) and visceral WAT based on its location (Chau et al. 2014). It is believed that visceral WAT is more responsible for obesity and metabolic syndromes.

Figure 1.1.1

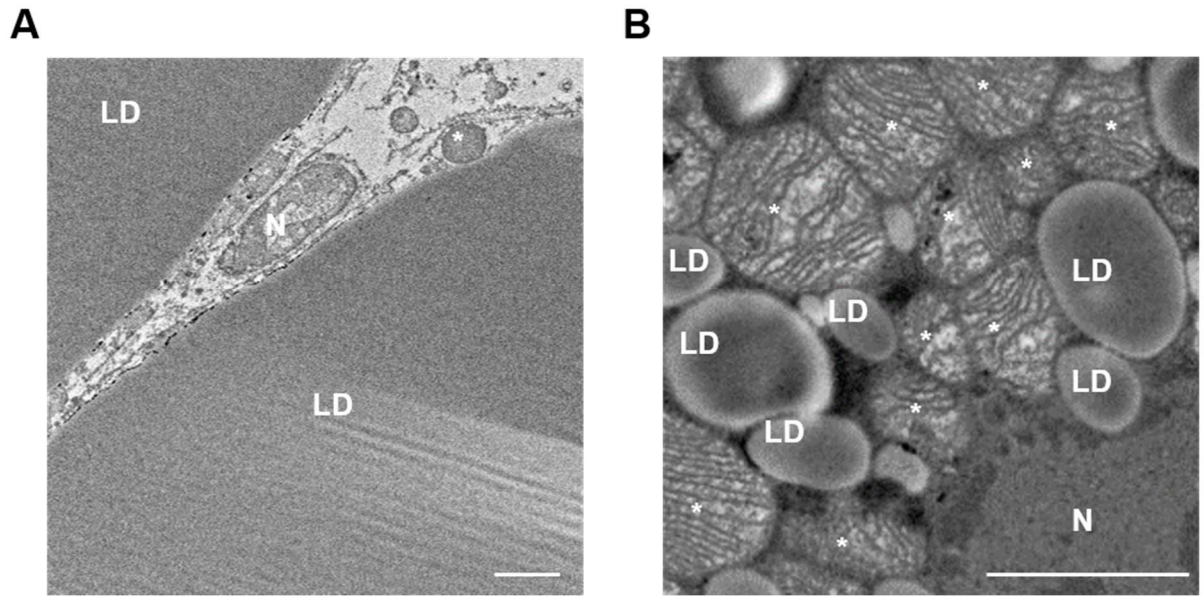


Figure 1.1.1 Subcellular structure of adipocytes.

Representative electron microscopy (EM) images of mouse (A) epididymal white adipose tissue and (B) interscapular brown adipose tissue. N, nucleus; LD, lipid droplet; asterisk, mitochondria. Scale bar: 2 μm.

1.1.2 Brown Fat

Brown adipocytes originate from Myf5-positive progenitors (Seale et al. 2008), and in adulthood also from endothelial precursors (Tran et al. 2012) and skeletal muscle stem cells (stellate cells) (Schulz et al. 2011). Patrick Seale and his colleagues led a series of studies showing that a transcriptional regulator PRD1-BF1-RIZ1 homologous domain containing 16 (PRDM16) determines the differentiation of brown adipocytes and is essential for maintaining BAT functions (Seale et al. 2007, Kajimura et al. 2008, Seale et al. 2008, Chi and Cohen 2016). Brown adipocytes are featured by numerous smaller LDs, considerable cytoplasm, and a large amount of mitochondria (Fig.1.1.1B). There are abundant capillaries in BAT to provide oxygen and distribute heat.

Abundant in newborns and hibernating mammals, BAT dissipates energy and generates body heat by non-shivering thermogenesis. This process depends on a membrane protein uncoupling protein 1/thermogenin (UCP1) (Matthias et al. 2000). UCP1 increases the inner mitochondrial protein permeability, decreases the mitochondria proton gradient, and thus enables the energy from nutrient (e.g. FA) oxidation to generate heat rather than synthesize adenosine triphosphate (ATP). In human studies, enhanced cold-induced BAT activity is correlated with less adiposity and better glucose metabolism (van Marken Lichtenbelt et al. 2009, Matsushita et al. 2014). Therefore, BAT is considered an intriguing target of anti-obesity and anti-diabetes therapies.

1.1.3 Beige Fat

Early studies reported that some brown adipocytes appeared in WAT, which were later named as beige/brite adipocytes (Harms and Seale 2013). Though located in WAT and sharing the same

origin with white adipocytes, beige adipocytes have multilocular LDs, high mitochondrial content and brown-specific gene expression including UCP1. In response to cold or β-adrenergic agonists, beige adipocytes are induced and demonstrate thermogenic capacity (Bartelt and Heeren 2014). Stimulating the activity of beige adipocytes may offset the dysregulated WAT and improve the status of metabolic diseases. There is also emerging research interest in the interconversion between white adipocytes and beige adipocytes (Cinti 2009). The white-to-brown conversion in fat is often called “browning”.

Besides adipocytes, there are other cell types in adipose tissue contributing to adipose growth and function, including preadipocytes, macrophages, lymphocytes, fibroblasts and vascular cells. Preadipocytes, also known as adipose progenitor cells, are important in controlling adipocyte number especially in context of obesity and diabetes (Rodeheffer et al. 2008). Macrophage accumulation in fat tissue contributes to systemic inflammation and insulin resistance (Weisberg et al. 2003, Xu et al. 2003). In WAT most of the infiltrating macrophages surround dead adipocytes and form crown-like structures (Murano et al. 2008).

1.2 Adipogenesis

Adipogenesis is the differentiation process of fibroblast-like preadipocytes into lipid-rich, insulin-responsive, and adipokine-secreting adipocytes. Adipogenesis has been widely studied since 1970s (Green and Kehinde 1975), and people's understanding of this complicated, integrated process is still developing. Most of the knowledge about adipogenesis comes from *in vitro* models which partially correlates with adipose development *in vivo* (Soukas et al. 2001). This process is generally described as two phases: the determination phase in which pluripotent stem cells convert into preadipocytes and lose the potential to differentiate into other types of cells (e.g. osteogenesis, chondrogenesis, neurogenesis, angiogenesis), and the terminal differentiation phase in which preadipocytes gradually acquire the morphology and function of mature adipocytes. The terminal differentiation phase is studied more since some of the cellular models of adipogenesis are preadipocyte lineages without the multipotency to differentiate into other types. The differentiation process involves a set of transcriptional regulation, and the most important regulators include peroxisome proliferator-activated receptor-gamma (PPAR-γ) (Brun et al. 1996) and CCAAT-enhancer-binding proteins (C/EBPs) (Darlington et al. 1998). A mixture of hormonal inducers are usually applied to induce adipogenesis in cellular models (Rosen and MacDougald 2006). 3T3-L1, a mouse preadipocyte cell line is the most common model to study adipogenesis *in vitro*. Adipose-derived stromal cells, bone marrow stromal cells, embryonic stem cells, and mouse embryonic fibroblasts (MEFs) have also been used for similar purpose as primary cell culture models, though the differentiation requirement and time varies (Ruiz-Ojeda et al. 2016).

1.3 Autophagy in Adipose Tissue

1.3.1 Overview of Autophagy

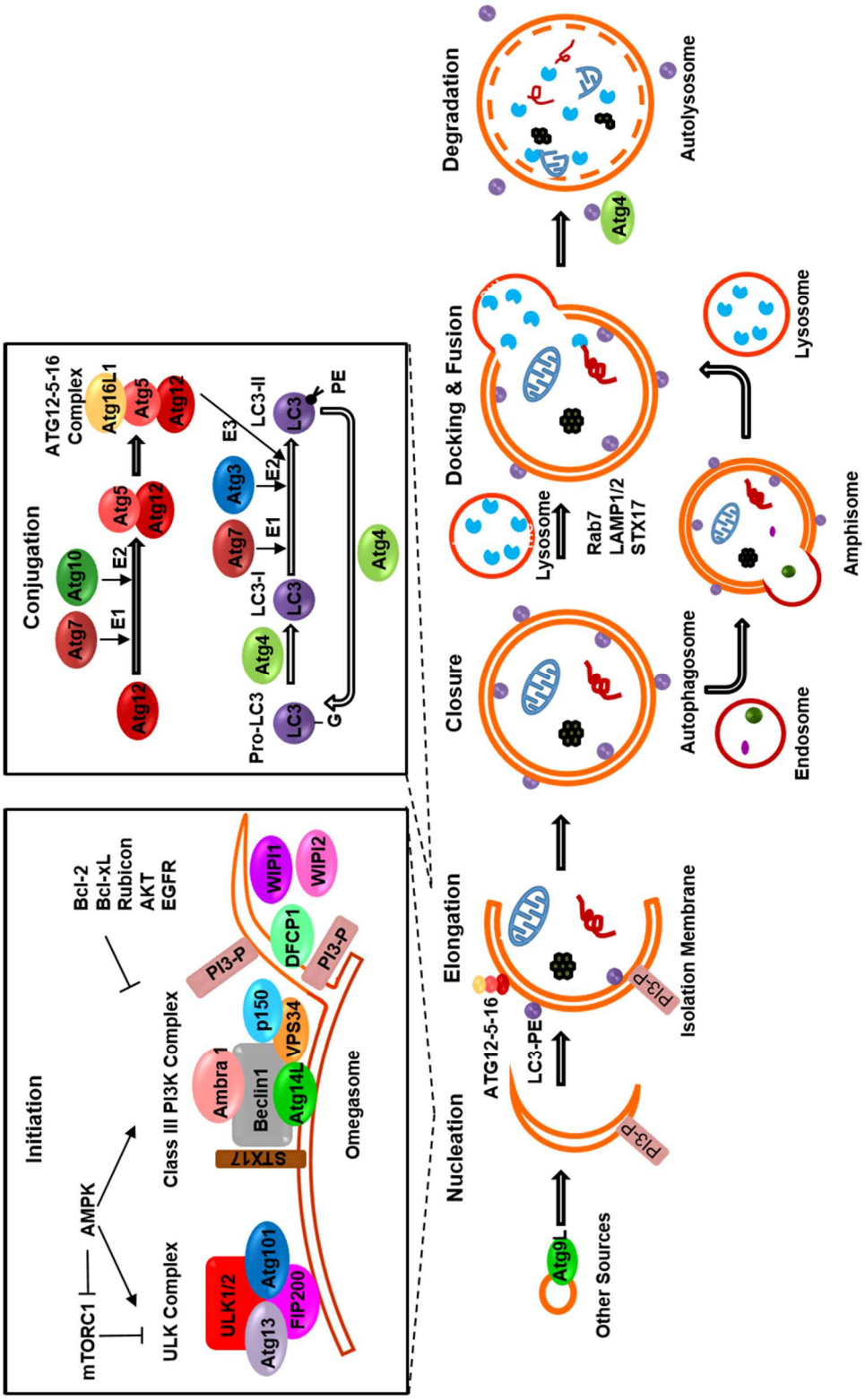
Autophagy is a highly conserved, genetically programmed lysosomal degradation pathway to maintain cellular homeostasis. Depending on the delivery routes of cargo there are three types of autophagy: marcoautophagy, microautophagy, and chaperon-mediated autophagy (CMA).

Microautophagy is constitutively active and autophagy gene-independent, which directly traps cytoplasmic components by invagination or budding of the surface of lysosomes (in mammals) /vacuoles (in yeast) (Mijaljica et al. 2011, Li et al. 2012). Microautophagy is usually non-selective but can be selective in the case of micropexophagy (Farre and Subramani 2004), piecemeal microautophagy of the nucleus (micronucleophagy) (Roberts et al. 2003), and micromitophagy (Lemasters 2014).

CMA is chaperon-dependent selection of cytosolic proteins targeted for lysosomal degradation without formation of additional vesicles (Kaushik and Cuervo 2018). KFERQ-like motif-bearing proteins are recognized and delivered to lysosomes by chaperon heat shock cognate 71 kDa protein (HSC70) and other cochaperons. Their internalization in lysosomes depends on lysosome-associated membrane protein 2A (LAMP2A), which is identified in birds and mammals (Gough et al. 1995).

Macroautophagy (hereafter referred to as autophagy) involves the formation of a double-membrane autophagic vesicle. Autophagy is regulated by more than 40 autophagy-related (Atg) genes that have been so far identified, which generally involves the following key steps as illustrated in Figure 1.3.1.

Figure 1.3.1



(See figure legend on next page.)

Figure 1.3.1 Schematic of the autophagy process.

Autophagy is tightly regulated by a series of autophagy-related genes. Upon nutrient deprivation, mTOR inhibition or increased activity of AMPK activates the ULK1 complex followed by activation of the Beclin 1/VPS34 complex. This results in PI3-P enrichment in the autophagosome initiation site and recruitment of additional Atg proteins. Atg9L delivers membranes from other cell organelles, such as Golgi bodies, to supply the membranes. The double membrane or isolation membrane expands and encloses cell organelles, misfolded proteins, or xenobiotics. The two ubiquitin systems, the Atg7-Atg3-Atg8/LC3 and Atg12-Atg5-Atg16L1 complexes, as well as Atg4 regulate the lipidation of LC3, which is essential for membrane elongation. The autophagosomes fuse with lysosomes to form autolysosomes, and this process is primarily mediated by Rab7, lysosome-associated membrane protein 1/2, STX17. The enwrapped cargos in autophagosomes then are degraded in the autolysosomes by lysosomal hydrolases. The outer membrane LC3 is de-conjugated by Atg4, while the inner membrane LC3 is digested together with autophagy cargo in autolysosomes. G, glycine; E1, E1 ligase; E2, E2 ligase; E3, E3 ligase.

1) Initiation, a process that is regulated by the complex that includes Unc-51 like kinase 1 (ULK1)-FAK family-interacting protein of 200 kDa (FIP200)-Atg13. This complex is negatively regulated by the nutrient sensor the mechanistic target of rapamycin complex 1 (mTORC1) and positively regulated by the energy sensor Amp-activated protein kinase (AMPK) (Chang and Neufeld 2009, Hosokawa et al. 2009, Egan et al. 2011, Ding 2015).

2) Nucleation, which requires the endoplasmic reticulum (ER)-resident SNARE protein syntaxin 17 (STX17) that further recruits Atg14L to the rough ER or ER-mitochondria contact site, and Atg14L then recruits Beclin 1 and Vps34 to the autophagosome initiation site on the rough ER (Matsunaga et al. 2010, Hamasaki et al. 2013). Vps34 is the mammalian class-III PI3 kinase that promotes the generation of phosphatidylinositol 3-phosphate (PI3-P), which further recruits PI3-P effectors such as double FYVE domain-containing protein 1 (DFCP1), WD-repeat interacting protein with phosphoinositide 1 (WIPI1) and WIPI2 to initiate the biogenesis of autophagosomes (Proikas-Cezanne et al. 2004, Axe et al. 2008, Polson et al. 2010). Activating molecule in Beclin1-regulated autophagy (Ambra1) (Fimia et al. 2007), UV irradiation resistance-associated gene (UVRAG) (Liang et al. 2006), and Bif-1/Endophilin B1 (Takahashi et al. 2007) positively regulate this complex, whereas Bcl-2 (Pattingre et al. 2005), Bcl-xL, Run domain protein as Beclin 1 interacting and cysteine-rich containing (Rubicon) (Zhong et al. 2009, Sun et al. 2011), AKT, and epidermal growth factor receptor (EGFR) negatively regulate this complex;

3) Elongation, as the two ubiquitin-like conjugation systems, Atg7 (E1-like)-Atg3 (E2-like)- and the Atg12-Atg5-Atg16L1 complex (E3 ligase-like) regulate conjugation of phosphatidylethanolamine with microtubule-associated light chain (LC3) (called LC3-II), which expands the autophagosome membrane (Mizushima et al. 1998, Ichimura et al. 2000, Ohsumi 2001). Atg9, the only Atg protein that has transmembrane domains, also delivers membranes from

trans-Golgi network/endosomes to the site of autophagosome biogenesis in an ULK1-and Vps34-dependent manner to promote the elongation of the autophagosome membrane (Young et al. 2006). It should be noted that the Atg12-Atg5-Atg16L complex generally transiently attaches to the autophagosomal membranes and is later dissociated from the autophagosomal membranes (Itakura and Mizushima 2010). This is in contrast with LC3-II that is relatively stable on the autophagosome membranes till the fusion of the autophagosomes with lysosomes.

4) Closure, the mechanisms by which the autophagsome membranes fuse with each other and eventually form a complete enclosed double membrane vesicle are still not completely understood yet. However, work from Dr. Ohsumi's lab has shown that LC3-II protein has the hemifusion function *in vitro*, which may help to tether the autophagosome membranes resulting in eventual closure of the autophagosome (Nakatogawa et al. 2007). It should also be noted that PI3-P is also dephosphorylated locally by the phosphatases myotubularin-related protein 3 (MTMR3, also called Jumpy) upon closure of the autophagosomes (Vergne et al. 2009, Taguchi-Atarashi et al. 2010).

5) Finally, autophagosomes fuse with lysosomes to form autolysosomes, which is mediated by Ras-related protein 7 (Rab7), LAMP1/2 and STX17 (Jager et al. 2004, Huynh et al. 2007, Itakura et al. 2012). After fusion, the outer membrane of LC3-II is deconjugated from the autolysosomal membrane by Atg4B, and the inner membrane LC3-II is degraded together with autophagosome cargos by lysosomal proteases (Mizushima et al. 2002, Tanida et al. 2004).

Depending on the targets, autophagy can be categorized into non-selective bulk autophagy and selective autophagy. Non-selective autophagy breaks down proteins and organelles to provide the cell with nutrients in response to starvation (Mizushima 2007). Selective autophagy removes damaged and excess organelles as well as protein aggregates using specific receptors in both

nutrient-sufficient and poor conditions as well as other pathophysiological conditions such as exposure to xenobiotics or drugs (Ding and Yin 2012, Stolz et al. 2014, Williams et al. 2015, Ni et al. 2016, Yang et al. 2016). It should be noted that the different types of autophagy mentioned above can coexist in cells and are not mutually exclusive.

Due to the complex dynamic nature of the autophagic process, it is very challenging to monitor autophagy in a quantitative way, in particular *in vivo* in whole animal tissues/organs. While LC3-II is widely used to monitor the autophagic process, LC3-II itself is also degraded in the autolysosomes. Thus an autophagic flux assay, which monitors LC3-II levels with or without a lysosomal inhibitor such as chloroquine (CQ) or bafilomycin A1 (BAF), has been recommended to determine autophagy status by the autophagy research community (Klionsky et al. 2016). In addition, the level of p62/sequestosome 1 (SQSTM1) has also been suggested to use as another marker for autophagic flux because p62/SQSTM1 is normally degraded in response to starvation and accumulated in genetic autophagy gene deleted mouse livers (Komatsu et al. 2010, Ni et al. 2012). However, the levels of p62/SQSTM1 may not always be suitable to monitor autophagic flux because its levels are also regulated at the transcriptional level, which is often induced in many experimental autophagy models including prolonged starvation conditions (Sahani et al. 2014).

1.3.2 Autophagy and Lipid Metabolism

LD formation is an indispensable step of lipid metabolism. The Atg8/LC3 conjugation system is essential for autophagosome membrane formation but some evidence suggests that Atg8/LC3 conjugation is also involved in LD formation. Indeed, LD formation is suppressed in autophagy-

deficient hepatocytes (Shibata et al. 2009). As discussed above, the ubiquitin-like protein Atg8 also mediates the tethering and hemifusion of liposomes (Nakatogawa et al. 2007). However, it is not clear whether Atg8/LC3 is required for LD-LD fusion. Using Atg5 knockout (KO) and Atg7 KO MEFs that were loaded with oleic acid, we found that LD still formed in Atg5 KO and Atg7 KO MEFs and there was no difference for the number of LDs between wild-type (WT) cells and these KO cells. These data clearly indicate that LC3-II is dispensable for the LD formation (Li et al. 2018).

Notably, TGs and cholesterol in LDs could also be taken up by autophagosomes and delivered to lysosomes for degradation by acidic hydrolases through selective autophagy. This alternative pathway of lipid metabolism has been described and termed as lipophagy (Singh et al. 2009, Singh and Cuervo 2012), in contrast to the usual pathway via cytoplasmic neutral hydrolases. Autophagic removal of excess hepatic LDs has been shown in mice that were fed with high fat diet or challenge with alcohol, two common causes that induce non-alcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (Singh et al. 2009, Ding et al. 2010). Impaired lipophagy in liver exacerbates fatty liver disease (Martinez-Lopez and Singh 2015). In contrast, activation of autophagy attenuates fatty liver in both NAFLD and alcoholic fatty liver disease (Ding et al. 2010, Lin et al. 2013, Park et al. 2014). How autophagy selectively removes excess LDs is still largely unknown. A recent study showed that the small guanosine triphosphatase (GTPase) Rab7 is indispensable for LD breakdown in hepatocytes subjected to nutrient deprivation. Starvation activates Rab7 that promotes trafficking of both multivesicular bodies and lysosomes to the LD surface during lipophagy, resulting in the formation of a lipophagic synapse. Genetic knockdown of Rab7 leads to gross morphological changes of multivesicular bodies, lysosomes, and autophagosomes, resulting in the attenuation of hepatocellular lipophagy (Schroeder et al. 2015).

Future studies are needed to determine whether other autophagy receptor proteins such as p62/SQSTM1 are involved in selective lipophagy. Surprisingly, under starvation conditions, WT mice have increased accumulation of hepatic LDs, whereas liver autophagy-deficient mice (such as liver-specific Atg5, Atg7 and FIP200 KO) do not. These data suggest that some secondary effects in these mice may either affect the uptake of FAs from the blood or compensatory alternative LD breakdown pathways may be active in these autophagy-deficient mouse livers (Ma et al. 2013, Wang et al. 2014, Li et al. 2018).

In addition to autophagy (macroautophagy), CMA regulates LD biology and in the maintenance of lipid homeostasis. CMA specifically degrades the LD-associated proteins perilipin (PLIN) 2 and PLIN3 prior to lipolysis. AMPK phosphorylates PLIN2 and primes PLIN2 for its degradation by CMA (Kaushik and Cuervo 2016). Inhibition of CMA leads to decreased association of adipose triglyceride lipase (ATGL) and lipophagy-related proteins with LDs resulting in decrease in lipid oxidation and accumulation of LDs (Kaushik and Cuervo 2015). In addition to macroautophagy and CMA, lysosome itself can also directly envelop cargos for their degradation via microautophagy. However, the significance for microautophagy for LDs and lipid homeostasis is still largely elusive.

1.3.3 Autophagy and Adipogenesis *In Vitro*

Adipocytes derive from multipotent mesenchymal stem cells and require massive cytoplasmic remodeling. During adipogenesis of primary MEFs, the levels of LC3-II increase and p62/SQSTM1 protein decrease together with increased autophagic structures enclosing mitochondria and other organelles, indicating enhanced autophagy (Baerga et al. 2009). Emerging

evidence in cells and transgenic mice suggests that some genes critical in autophagy process affect mass, differentiation and physiological functions of adipose tissue. The notion is supported by the following evidence: 1) Atg5 or Atg7 deficiency impairs adipogenesis *in vitro* and *in vivo* (Baerga et al. 2009, Singh et al. 2009, Zhang et al. 2009); 2) treatment of CQ, an autophagy inhibitor that increases lysosomal pH, inhibits adipogenesis *in vitro* (Baerga et al. 2009, Singh et al. 2009); 3) mice with deletion of *p62*, an autophagy substrate, develop mature-onset obesity and insulin resistance (Rodriguez et al. 2006). Interestingly, upregulated autophagy proteins and genes have also been observed in human adipose tissue samples in obesity although no autophagic flux data are available in obese human adipose tissue (Kovsan et al. 2011). The role of autophagy in adipose tissue especially in disease context thus still needs to be further investigated.

Mechanistic target of rapamycin (mTOR), a highly conserved serine-threonine protein kinase, is a key regulator of cell growth and metabolism. As discussed above, mTOR complex 1 (mTOR1) is a negative regulator at the preinitiation complex to regulate the initiation of autophagosome biogenesis (Yang and Klionsky 2010). Pharmacological and genetic evidence has proved that mTORC1 is required for adipogenesis and adipose maintenance *in vitro* (Kim and Chen 2004, Polak et al. 2008). Activation of mTOR suppresses lipolysis, stimulates lipogenesis, and promotes fat storage (Chakrabarti et al. 2010). The phenotypes observed from the activation of mTOR in adipocytes would be difficult to reconcile with the phenotypes from the adipocyte-specific *Atg5* or *Atg7* KO mice because one would assume that activation of mTOR would lead to decreased autophagy. However, there are some distinctive differences here. Unlike adipocyte-specific *Atg5* or *Atg7* KO mice, activation of mTOR may not be able to completely eliminate autophagy. In addition, autophagy can also occur independent of mTOR (Li et al. 2016). Therefore whether autophagy is involved in mTOR-regulated adipocyte biology remains to be further investigated.

1.3.4 Autophagy and Remodeling of Beige Adipocytes

As briefly mentioned above, beige adipocytes, also called the browning of white fat, are recently discovered as a distinct type of thermogenic fat to exert anti-obesity effects by burning FA and glucose instead of storing lipids (Peirce et al. 2014). Both beige adipocytes and the traditional brown adipocytes are competent for thermogenesis through the actions of UCP1 and share similar morphological characteristics such as multilocular LDs and abundant mitochondria. Beige adipocytes are inducible and derived from white adipocytes in response to cold stress or pathways that elevate intracellular cyclic adenosine monophosphate (cAMP) (Bartelt and Heeren 2014). Interestingly, the "browning of white" process is reversible and beige adipocytes can return to white adipocytes by removing excess mitochondria likely via selective autophagy for mitochondria (mitophagy). Indeed, a recent report provides strong evidence to support that autophagy pathway is crucial for mitochondrial clearance during the beige-white transition. Adipocyte-specific *Atg5* or *Atg12* KO mice prevent beige adipocyte loss after withdrawing external stimuli. These mice also maintain high thermogenic capacity and protect against diet-induced obesity and insulin resistance (Altshuler-Keylin et al. 2016). Selective mitophagy is mediated by Pink1-Parkin pathway (Ding and Yin 2012, Ni et al. 2015, Pickrell and Youle 2015) but it remains unclear whether Pink1 or Parkin plays a role in mitophagy during the beige-white transition.

1.3.5 Genetic Approaches to Study Autophagy in Adipose Tissue

Since autophagy is involved in development and many biological processes, and whole-body autophagy KO may be lethal (Kuma et al. 2004), tissue-specific KO system is preferable if genetic autophagy inhibition is desired in autophagy study. The *Cre/LoxP* site-specific recombination system which inactivates specific genes in fat has been widely applied in mice to study adipocyte functions. A commonly used promoter is *adipocyte protein-2* (*aP2*) gene encoding fatty acid-binding protein-4 (Fabp4). However, the specificity of *aP2* deletion has not been satisfying, as Fabp4 also expresses in heart, skeletal muscle, and testis (Lee et al. 2013, Jeffery et al. 2014). The promoter cassette of *Adipoq* gene encoding adiponectin has been proved to be more specific for adipocytes (Wang et al. 2010), and have been used more frequently in recent years. Since UCP1 is predominantly expressed in BAT and brown adipocytes, *Ucp1-Cre* can be used in study focused in brown adipocytes and beige adipocytes (Guerra et al. 2001, Altshuler-Keylin et al. 2016). As discussed in 1.3.1, autophagy is tightly regulated by a series of genes. Up-to-date *in vivo* studies on adipose autophagy using transgenic models are summarized in Table 1. Since autophagy can be activated by mTOR inhibition, transgenic mice modulating mTOR in adipose tissue may also affect autophagy (Table 2).

Table 1 Phenotypes of transgenic mice modulating autophagy in adipose tissue

Gene	Knockout condition	Phenotypes	References
p62	Whole body	Mature-onset obesity; insulin resistance	(Rodriguez et al. 2006)
Atg5	Whole body	Fewer sWAT cells in neonatals pups	(Baerga et al. 2009)
Atg5, Atg12	*Ucp1-Cre*	Inhibited beige-to-white conversion; higher thermogenic capacity; resistance to diet-induced obesity and insulin resistance	(Altshuler-Keylin et al. 2016)
Atg7	*aP2-Cre*	Reduced body weight and WAT mass; increased multilocular adipocytes and mitochondria content in WAT; increased physical activity; increased insulin sensitivity; resistance to high fat diet (HFD)-induced obesity	(Singh et al. 2009, Zhang et al. 2009)
	Atg7(+/-) crossed with *ob/ob*	Aggravated insulin resistance; increased inflammasome activation	(Lim et al. 2014)
Bif1	Whole body	Obesity; insulin resistance; impaired lipid catabolism	(Polak et al. 2008, Liu et al. 2016)
Pik3c3	*aP2-Cre*	With aging: Reduced adiposity; improved glucose tolerance; increased browning	(Lee et al. 2016, Ghosh et al. 2018)

Table 2 Phenotypes of transgenic mice modulating mTOR in adipose tissue

Gene	Knockout condition	Phenotypes	References
Raptor	*aP2-Cre*	Reduced body weight and fat mass; increased browning; increased energy expenditure due to uncoupling respiration in WAT; improved metabolic parameters; resistance to HFD-induced obesity	(Polak et al. 2008)
	Adipoq-Cre	Reduced fat mass with aging; fatty liver; insulin intolerance; resistance to HFD-induced obesity but more severe hepatic steatosis; impaired lipid absorption	(Lee et al. 2016)
TSC1	*aP2-Cre*	Whitening in BAT	(Xiang et al. 2015)
	Adipoq-Cre	Reduced visceral fat adiposity; increased browning; increased diet-induced glucose tolerance	(Magdalon et al. 2016)
mTOR	*Adipoq-Cre*	Reduced fat mass; impaired BAT development; increased browning; impaired insulin resistance	(Shan et al. 2016)

To study adipocyte function *in vitro*, researchers have used primary MEFs and adipose-derived stromal vascular fraction cells (SVFs) isolated from mice. These are preadipocytes, i.e. they can be differentiated into adipocyte-like cells under the exposure to hormonal inducers. Primary preadipocytes isolated from KO mice are a powerful tool to genetically modulate autophagy in studying adipogenesis. However, these cells lose the adipogenic potential and become senescent after a few passages.

3T3-L1 cell line is another type of mouse preadipocytes that is commonly used for the studying of adipogenesis *in vitro* (Green and Kehinde 1975). They can be passed for over 10 passages, and are more suitable for small interfering RNA (siRNA) or small hairpin RNA (shRNA) transfections, but not suitable for adeno-associated virus (AAV) infection unless coxsackie and adenovirus receptors (CARs) are introduced (Orlicky and Schaack 2001). Abundant evidence proves that autophagy and mTOR signaling is essential for adipogenesis *in vitro* (Table 3 & 4).

Table 3 *In vitro* adipogenesis studies genetically modulating autophagy

Gene	Cell type	Phenotypes	References
p62	-/- pMEFs	Enhanced adipogenesis	(Rodriguez et al. 2006)
Atg5	3T3-L1 cells	Impaired adipogenesis	(Singh et al. 2009)
	-/- or +/- pMEFs	Impaired adipogenesis	(Baerga et al. 2009, Zhang et al. 2013)
Atg7	3T3-L1 cells	Impaired adipogenesis	(Singh et al. 2009)
	-/- pMEFs	Impaired adipogenesis	(Zhang et al. 2009)
Atg4b	3T3-L1 cells	Impaired differentiation	(Guo et al. 2013)
Ulk1, Ulk2	3T3-L1 cells	Decreased mitochondria respiration and ATP production; opposing effect on lipid metabolism and glucose uptake	(Ro et al. 2013)

pMEFs: Primary mouse embryonic fibroblast

Table 4 *In vitro* adipogenesis studies genetically modulating mTOR

Gene	Cell type	Phenotypes	References
Raptor	3T3-L1 cells	Impaired adipogenesis and adipose maintenance; increased lipolysis	(Polak et al. 2008)
Tsc2	-/- pMEFs	Enhanced adipogenesis	(Chakrabarti et al. 2010)
Tsc1	Brown preadipocytes from *aP2-Cre* KO mice	Brown-to-white conversion	(Xiang et al. 2015)
mTOR	Brown preadipocytes from *Adipoq-Cre* KO mice	Impaired differentiation	(Shan et al. 2016)

1.4 Alcohol-induced Multi Organ Injury

Chronic or acute alcohol abuse often leads to liver injury associated with alcoholic hepatitis, liver fibrosis, cirrhosis and liver cancer (Gao and Bataller 2011). In addition to the liver, alcohol abuse also induces a variety of other tissue injuries including pancreatitis (Gukovsky et al. 2008, Gu et al. 2013), cardiomyopathy (Ren and Wold 2008, Piano and Phillips 2014), neurotoxicity (Harper 2009), muscle loss (Lang et al. 2004), impaired immune functions (Gao and Bataller 2011), endocrine and fetal abnormalities (Pruett et al. 2013), and osteoporosis (Maurel et al. 2012). According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), more than 18 million adults are affected by alcoholism in the United States, which costs 27 billion dollars for treating alcohol-attributable diseases.

The mechanisms for alcohol-induced detrimental effects in various tissues/organs have been extensively studied, which involves genetic and environmental factors as well as altering multiple cellular signaling pathways. These mechanisms involve ethanol and its metabolites that induce reactive oxygen species (ROS) generation, lipid peroxidation, cytokine expression/inflammation, organelle damage and stress, and activate both apoptotic and necrotic cell death pathways (Molina et al. 2002). However, the full picture, especially the cellular adaptive and protective mechanisms against ethanol-induced stress and tissue injury have not been well-depicted yet. Currently, the treatment for chronic alcohol diseases is not very effective owing largely to our incomplete understanding of the cellular adaptive response to ethanol toxicity.

Accumulating evidence has shown that altered autophagy is implicated in the pathogenesis and protection of ethanol-induced tissue injury (Ding et al. 2011, Dolganiuc et al. 2012, Czaja et al. 2013). This section aims to summarize current knowledge about the role of autophagy in

alcohol-induced injury in multiple tissues/organs and their underlying molecular mechanisms. Potential therapeutic targets based on modulation of autophagy will also be discussed.

1.4.1 Alcohol Metabolism and Its Effect on Tissue Injury

Ethanol is metabolized through several pathways. Predominantly alcohol is metabolized by alcohol dehydrogenase (ADH) into acetaldehyde, a highly toxic byproduct, and then by aldehyde dehydrogenase (ALDH) into acetate, a less harmful substance. The most important two isoforms of ALDH is the cytosolic ALDH1 and the mitochondrial ALDH2 (Crabb et al. 2004). The oxidation mainly occurs in liver (Lieber et al. 1975), but also in pancreas (Vonlaufen et al. 2007), heart (Ren and Wold 2008), and other organs (Zakhari 2006), accompanied by conversion of nicotinamide adenine dinucleotide (NAD^+) into its reduced form, NADH, which plays several critical roles in redox metabolism. Besides, ethanol can also be metabolized by cytochrome P450 family 2, subfamily E, polypeptide 1 (CYP2E1) and catalase. Excessive ethanol exposure largely induces CYP2E1, which not only mediates and activates reactions of many toxicological substrates, but also generates ROS leading to cellular damage (Lu and Cederbaum 2008). Additionally, ethanol could interact with FA and generate FA ethyl ester (FAEE) catalyzed by FAEE synthase in many tissues and organs in a non-oxidative pathway (Zelner et al. 2013). FAEE was thought to have a minor effect and mainly considered as a diagnostic marker, but accumulated evidence shows that FAEE exacerbates injury after ethanol exposure especially in pancreas (Werner et al. 2002, Wu et al. 2008), liver (Wu et al. 2006) and heart (Beckemeier and Bora 1998, Wu et al. 2006, Wu et al. 2008), and is facilitated by ADH deficiency (Bhopale et al. 2006). One explanation of cytotoxicity of FAEE is that FAEE binds to mitochondria membrane and with its

hydrolysis products, FAs, causes damage by uncoupling oxidative phosphorylation (Lange and Sobel 1983).

Alcohol is easily absorbed and could be metabolized and impact on almost all over the body. Matured brain is less affected compared with developing brain due to the blood-brain barrier, but excessive oxidative stress and intracellular Ca^{2+} release induced by ethanol could impair the barrier function (Haorah et al. 2007). Dysregulated metabolites lead to changes in carbohydrate metabolism, cell death signaling, mitochondria permeability transition and epigenetic regulation (Zakhari 2013). Apoptosis and necrosis induced or exacerbated by alcohol metabolism have been studied in liver (Nanji and Hiller-Sturmhofel 1997, Ishii et al. 2003), pancreas (Gukovskaya et al. 2006, Wang et al. 2006), heart, brain (mostly developing brain) (Johansson et al. 2009, Alfonso-Loeches et al. 2010) (Olney et al. 2002) and skeletal muscle (Fernandez-Sola et al. 2003). Recent evidence has also showed the involvement of necroptosis, the receptor interacting protein (RIP)-mediated non-apoptotic cell death pathway in the pathogenesis of alcoholic liver disease (ALD) (Roychowdhury et al. 2013). The effect of metabolites in different tissues/organs will be further discussed below.

1.4.2 Autophagy in Alcohol-induced Liver Injury

ALD is a major cause of morbidity and mortality worldwide. The spectrum of ALD ranges from steatosis, inflammation, fibrosis, and cirrhosis to hepatocellular carcinoma (Altamirano and Bataller 2011, Williams et al. 2014). In liver, ethanol is mainly metabolized by ADH, catalase and CYP2E1, which promotes the generation of ROS and other reactive toxic metabolites leading to tissue injury (Lieber et al. 1975, Lu and Cederbaum 2008).

Notably, liver plays a key role in lipid metabolism, and hepatocytes take up free fatty acids (FFAs) and convert them to TGs for storage with cholesterol in LDs (Martinez-Lopez and Singh 2015). Several mechanisms could contribute to ethanol-induced steatosis: enhanced lipid synthesis by activated transcription factor sterol regulatory element binding protein-1 (SREBP-1), decreased FA oxidation by downregulated nuclear receptor PPARα or mitochondria damage, decreased very low density lipoprotein (VLDL) secretion, promoting effect of activated transcription factor early growth response-1 (Egr-1), and changed levels of important humoral factors including adiponectin and tumor necrosis factor alpha (TNF-α) (Donohue 2007).

Liver is one of the most active organs, which plays a central role in regulating the overall organism energy balance by controlling carbohydrate and lipid metabolism. Under physiological conditions, liver also serves as a major buffering system to ensure other tissues to function normally by maintaining the homeostasis of macro- and micronutrients. To accomplish these vital missions, liver may rely on autophagy, the cellular catabolic process to breakdown macromolecules, lipids and damaged/excess organelles. Indeed, liver-specific autophagy gene KO mice have disrupted metabolism of proteins, glucose and lipids, accumulated damaged and excess organelles such as mitochondria and peroxisomes, and results in increased cell death, inflammation, and liver tumorigenesis (Singh et al. 2009, Komatsu et al. 2010, Takamura et al. 2011, Ni 2014). ALD also involves the disruption of cellular metabolism of proteins, lipids and homeostasis of organelles such as mitochondria and ER resulting in increased cell death that contributes to alcoholic hepatitis, liver fibrosis, cirrhosis and liver cancer (Gao and Bataller 2011, Williams et al. 2014). All these pathogenic events are intimately related to the autophagic process, and modulating autophagy may thus affect the ALD pathogenesis. Indeed, accumulating evidence

now indicates that autophagy plays a critical role in the pathogenesis of ALD (Ding et al. 2011, Dolganiuc et al. 2012, Czaja et al. 2013).

Differential Autophagy Status in Acute and Chronic ALD Models

Many animal models as well as *in vitro* cell culture models have been developed to study ALD. While baboons and pigs have been used to study ALD, rodents have been predominantly used in current ALD because of its lower cost and propensity for genetic modulation (Arteel 2010). The animal models for ALD include acute alcohol gavage (Carson and Pruett 1996), *ad libitum* oral alcohol in drinking water (Best et al. 1949), intragastric infusion (Tsukamoto-French model) (Tsukamoto et al. 1985), chronic Lieber-DeCarli diet ethanol feeding (DeCarli and Lieber 1967), and the most recent chronic-plus-binge model (Bertola et al. 2013).

There are some controversial reports regarding the autophagy status in ALD research. The reasons behind these controversial reports are likely due to the complexity of autophagy assays and the use of many different ALD models. It is generally agreed that acute ethanol (binge) treatment increases autophagy in mouse livers and in primary cultured murine hepatocytes, a conclusion that is supported by autophagic flux data in these studies (Ding et al. 2010, Ni et al. 2013). In contrast, impairment of autophagy has also been reported in ethanol-treated CYP2E1 overexpressing HepG2 cells or in CYP2E1 knockin mice that were given acute alcohol twice a day for four days (Wu et al. 2010, Wu et al. 2012). However, only a decreased LC3-II/I ratio was observed and no autophagic flux assays were conducted in these studies. Using HepG2 cells that stably overexpressed both CYP2E1 and ADH, Thomes *et al* (Thomes et al. 2013) reported that ethanol treatment increased autophagosome synthesis but also impaired lysosomal degradation. However, the autophagic flux data in this study actually supported an increase in autophagy

(Thomes et al. 2013). Early works from the same group showed that ethanol treatment may increase lysosomal pH and impair cathepsin maturation (Kharbanda et al. 1995, Kharbanda et al. 1997). Thus it is possible that increased autophagy by ethanol treatment may serve as a compensatory mechanism in response to ethanol-induced mild impaired lysosomal functions. In contrast to acute alcohol treatment, it has been generally thought that chronic alcohol consumption may have impaired autophagy in the liver because it has long been shown that chronic alcohol administration leads to hepatomegaly and protein accumulation in liver (Baraona et al. 1975, Donohue et al. 1989, Donohue 2009). In a chronic ethanol feeding study, Lin *et al* performed autophagic flux studies in mice that were fed an ethanol diet for 4 weeks and increased autophagic flux was found in that study (Lin et al. 2013). However, we should interpret these data cautiously since the autophagic flux assay was only performed at one time point after the 4 week feeding, while ideally, autophagic flux assays should be applied to multiple time points owing to the dynamic nature of autophagy. In addition, the autophagy status in other ALD models, such as the Tsukamoto-French model and the chronic-plus-binge model, has not been reported. Recent work from our lab showed that chronic feeding plus acute binge ethanol impaired transcription factor EB (TFEB), a master regulator of lysosomal biogenesis, resulting in decreased number of hepatic lysosomes and insufficient autophagy (Chao et al. 2018). Moreover, in addition to hepatocytes, whether and how ethanol would affect autophagy on other liver cells, such as hepatic stellate and Kupffer cells, and their impacts on ALD are largely unknown. Regardless of the controversies on autophagy status, it has been unanimously shown that activating autophagy is beneficial against ALD in various ALD models (Ding et al. 2010, Lin et al. 2013, Ni et al. 2013, Yang et al. 2014).

Possible Mechanisms Affecting the Autophagy Process Induced by Alcohol

As discussed above, autophagy is a dynamic multi-step process that is tightly regulated by many signaling pathways involving nutrients, energy and stress response. Below we discuss the regulating pathways of autophagy that have been shown to be affected by alcohol.

Class I PI3K-Akt-mTOR

As discussed above, mTORC1 is a negative regulator at the pre-initiation complex to regulate the initiation of autophagosome biogenesis (Yang and Klionsky 2010). mTOR is part of two structurally and functionally different complexes, mTORC1 and mTORC2. The former complex is sensitive to rapamycin and plays a major role in regulation of cell growth and autophagy. mTORC1 is also a sensor of various signals including growth factors, insulin, nutrients, energy status and cellular stressors. In nutrient-sufficient condition, growth factors activate the class I phosphoinositol-3-kinase (PI3K) to catalyze PIP3 and subsequently activate Akt, which then further activates downstream mTORC1 and inhibits autophagy. mTORC2 may also negatively regulate autophagy because it is required for full activation of Akt (Facchinetti et al. 2008, Ikenoue et al. 2008). Suppression of Akt and mTOR are common mechanisms of autophagy induction, which is also affected by acute ethanol treatment. It has been demonstrated that acute ethanol-treated mouse liver and chronic ethanol-treated rat liver has increased expression of phosphatase and tensin homolog (PTEN) resulting in the suppression of Akt (Yeon et al. 2003, He et al. 2007). We also found that acute ethanol treatment decreases the level of phosphorylated Akt in mouse liver (Ni et al. 2013). Besides, ethanol treatment inhibits mTORC1 activity in primary cultured mouse hepatocytes (Ding et al. 2010). More importantly, pharmacological inhibition of mTOR by either rapamycin or Torin 1 significantly suppresses acute ethanol-induced liver steatosis and injury (Ding et al. 2010, Czaja et al. 2013). More future studies are needed to further determine

the detailed time-course changes of mTOR and their associations with autophagy during chronic alcohol feeding.

Interestingly, application of wortmannin, a specific PI3K/Akt inhibitor showed dual effects on acute ethanol-induced fatty liver depending on dose (Zeng et al. 2012). PI3K/Akt activation is known to enhance sterol regulatory element-binding protein-1 (SREBP-1)(Porstmann et al. 2008), and SREBP-1 coordinates autophagy-lysosomal activities and lipid metabolism (Jegga et al. 2011). In this model, low dose of wortmannin alleviated the rise of hepatic TG possibly by inhibiting SREBP-1 via PI3K/Akt inhibition, while high dose of wortmannin aggravated the disease likely due to the block of autophagy. This suggests that special attention should be paid to the interpretation of use of PI3K/Akt inhibitor in alcohol-induced fatty liver models.

AMPK

AMPK is a key energy sensor that regulates cellular metabolism and energy homeostasis. AMPK can directly inhibit mTOR through increased phosphorylation of TSC2 (Inoki et al. 2003, Kim et al. 2011) and Raptor (Gwinn et al. 2008), which activates autophagy. AMPK also promotes autophagy by phosphorylating ULK1, Vps34 and Beclin1 (Egan et al. 2011, Kim et al. 2011, Kim et al. 2013). However, administration of an AMPK activator adenosine, 5-amino-4-imidazole carboxamide riboside (AICAR) suppresses autophagy in hepatocytes (Samari and Seglen 1998). Moreover, administration of compound C, an AMPK inhibitor, activates autophagy via AMPK-independent blockade of the Akt/mTOR pathway, which overcomes the expected inhibitory effect on autophagy via AMPK inhibition in cancer cells (Vucicevic et al. 2011). Several lines of evidence show that AMPK activity is reduced in liver by ethanol consumption, which is believed to promote fatty liver through activation of SREBP-1 and up-regulation of lipin-1 expression (You

et al. 2004, Hu et al. 2012). The exact role of AMPK in ethanol-induced autophagy is not clear but it is possible that ethanol induces autophagy independent of AMPK activation.

ADH, CYP2E1 and ROS

In liver, ethanol is mainly metabolized by ADH and CYP2E1, which promotes the generation of ROS and other reactive toxic metabolites. Interestingly, ethanol-induced autophagy requires its metabolism and ROS production because autophagic flux was only induced in HepG2 cells stably expressing ADH and CYP2E1 but not in parental HepG2 cells (Ding et al. 2010, Thomes et al. 2013). Moreover, blocking ADH and CYP2E1 by 4-methylpyrazole or inhibiting ROS by antioxidants also reversed the inhibition of mTOR and diminished increased green fluorescent protein (GFP)-LC3 puncta (Ding et al. 2010, Thomes et al. 2013). It seems that alcohol oxidation by CYP2E1 is also important for alcohol-induced inhibition of cellular proteasome activity and increased autophagosome numbers (Thomes et al. 2012). It has been proposed that ROS may activate autophagy through modulating the oxidization of Atg4, an autophagy machinery protein important for generating and recycling of LC3-II (Scherz-Shouval et al. 2007). As discussed above, studies from ethanol-treated HepG2 cells that are overexpressing Cyp2E1 and ethanol-treated *Cyp2e1* KO or knockin mice showed decreased LC3-II levels (Wu et al. 2010, Wu et al. 2012, Wu and Cederbaum 2013, Yang et al. 2014). While the authors concluded that CYP2E1-mediated metabolism of ethanol may lead to inhibition of autophagy in these studies, autophagic flux assays were not conducted in these studies. Future studies are needed to further confirm the autophagy status in ethanol-treated *Cyp2e1* KO or knockin mice by performing an autophagic flux assay.

FoxO3 and SIRT1

Forkhead box-containing protein class O (FoxO) family of DAF-16 like transcription factors are evolutionarily conserved transcriptional factors that regulate the expression of genes involved in multiple cellular functions including oxidative stress, glucose metabolism, apoptosis, cell cycle transition as well as DNA repair (Huang and Tindall 2007, Tikhanovich et al. 2013). Four FoxO proteins including FoxO1, FoxO3, FoxO4 and FoxO6 are found in mammals, which have redundant yet distinctive roles in regulating gene expression. While FoxO1, FoxO3 and FoxO4 are ubiquitously expressed in most tissues, FoxO6 is mainly expressed in neurons. Studies from gene KO mice show that *FoxO1* KO mice are embryonically lethal due to impaired angiogenesis but *FoxO4* KO mice are viable with decreased migration of vascular smooth muscle cells (Li et al. 2007). While *FoxO3* KO mice are also viable, female mice are infertile due to ovarian activation and they also have spontaneous T cell activation and lymphoproliferation with time (Lin et al. 2004). FoxO3 mainly regulates the expression of genes responsible for oxidative stress, apoptosis, cell cycle transition as well as DNA repair but FoxO1 is more important in regulating glucose and lipid metabolism. All the FoxO family proteins are subjected to multiple post-translational modifications, including phosphorylation, acetylation, methylation and ubiquitination (Tikhanovich et al. 2013). Akt-mediated phosphorylation of FoxO3 causes its nuclear exclusion and thus inactivates FoxO3. It seems that the acetylation of FoxO3 mainly regulates the specificity of a subset of FoxO3 target genes by increasing the expression of antioxidant genes and suppressing the expression of apoptosis genes in response to oxidative stress (Brunet et al. 2004), whereas methylation of FoxO3 at K270 results in loss of DNA binding (Xie et al. 2012).

Increasing evidence now suggests that FoxO family proteins can also regulate autophagy by three distinctive mechanisms: direct transcriptional regulation of Atg gene expression (Mammucari et al. 2007, Zhao et al. 2007), transcriptional regulation of glutamine synthetase

expression and increasing intracellular glutamine levels (van der Vos et al. 2012), and interaction of cytosolic FoxO1 with Atg7 independent of its transcription activity (Zhao et al. 2010).

Sirtuin 1 (Sirt1) belongs to the evolutionarily conserved sirtuin family, which are NAD-dependent class III protein deacetylases. There are 7 sirtuins (Sirt1-7) that have been identified in mammals, which have distinct cellular locations. Sirt1, 6 and 7 are mainly in the nucleus, Sirt2 is mainly in the cytosol and Sirt3, 4 and 5 are found in the mitochondria (Haigis and Sinclair 2010, Nakagawa and Guarente 2011). Sirt1 has a broad range of physiological functions including the control of aging, metabolism and gene expression by promoting the deacetylation of a variety of substrates from histones to non-histone proteins. Increasing evidence suggests that sirtuins also play roles in regulating autophagy. *Sirt1* KO mouse embryonic fibroblasts have decreased autophagy in response to starvation, which is accompanied by increased acetylated Atg5, Atg7 and LC3 proteins, although it remains unclear how increased acetylation of these proteins affects their functions on autophagy (Lee et al. 2008). In response to stress, cytosolic FoxO1 is dissociated from Sirt2 resulting in an increase of acetylated FoxO1. Acetylated FoxO1 then binds to Atg7 and promotes autophagy in some human cancer cells (Zhao et al. 2010). Adult-onset and long-term calorie restriction in mice increased Sirt1 expression in aged kidney and attenuated hypoxia-associated mitochondrial and renal damage by enhancing Bcl2/adenovirus E1B 19 kDa-interacting protein 3 (Bnip3)-dependent autophagy. This increased autophagy was found to be regulated by Sirt1-mediated FoxO3 deacetylation resulting in increased expression of Bnip3 under hypoxia conditions (Kume et al. 2010).

We recently demonstrated that acute ethanol treatment increased the expression of Atg genes in mouse liver and in primary cultured mouse and human hepatocytes, which was accompanied by increased hepatic nuclear accumulation of FoxO3 (Ni et al. 2013). Acute ethanol treatment

decreased the level of phosphorylated Akt, causing decreased FoxO3 phosphorylation at Ser253, which could account for increased nuclear FoxO3. As resveratrol could increase SIRT1 affinity for both NAD^+ and the acetylated substrate through allosteric interaction (Howitz et al. 2003), activation of Sirt1 by resveratrol increased deacetylation of FoxO3 and thus enhanced ethanol-induced expression of Atg genes. Moreover, we found that *FoxO3* KO mice had decreased expression of Atg genes and had increased steatosis and liver injury compared to wild type mice after acute ethanol treatment (Ni et al. 2013). These findings indicate that FoxO3-mediated autophagy plays a protective role against alcohol-induced steatosis and liver injury. It has been suggested that ethanol consumption may inhibit Sirt1 via increased NADH/NAD^+ ratio through its metabolism. This may lead to the inhibition of autophagy in the liver either through FoxO3-dependent or independent mechanisms. However, ethanol consumption can also inhibit Akt phosphorylation, which can lead to increased nuclear retention of FoxO3 and ultimately increased expression of autophagy genes. Thus it is possible that Akt-mediated FoxO3 nuclear retention would be more important or dominant in regulating the expression of Atg genes than Sirt1-mediated acetylation of FoxO3. Nevertheless, our results suggest that activation of Sirt1, such as by using resveratrol, can further enhance ethanol-induced FoxO3-mediated expression of Atg genes. More studies are definitely needed to determine whether other FoxO and sirtuin family proteins are also involved in autophagy in alcohol-induced liver injury.

Interestingly, Nepal et al. found a link between AMPK/FoxO3 and autophagy when they studied the protective effect of globular adiponectin (gAcrp) on liver cells from ethanol-induced apoptosis (Nepal and Park 2013, Nepal et al. 2014). The fat-derived hormone adiponectin is significantly protective in ALD (Mandal et al. 2010). They showed that gAcrp restored ethanol-induced suppression of autophagy genes including *Atg5* and autophagosome formation, which was

accompanied by FoxO3 translocation. Silencing FoxO3 or its upstream regulator AMPK (Greer et al. 2007) abrogated the restoration, indicating the importance of FoxO3 and AMPK in autophagy in ethanol-induced injury possibly via modulating *Atg5*, in addition to the mechanisms mentioned previously.

Methionine, SAM and Methylation

Methionine is a sulfur-containing essential amino acid that is important in biogenesis of cysteine, carnitine, taurine, lecithin, phosphatidylcholine, and other phospholipids. Catalyzed by methionine adenosyltransferase, a liver specific enzyme, methionine is metabolized into S-adenosylmethionine (SAM), which is a universal methyl donor. After transferring the methyl group, SAM becomes S-adenosylhomocysteine, which can be further converted to adenosine and homocysteine via S-adenosylhomocysteine hydrolase. Homocysteine is a source for generation of methionine through methionine synthase (MS) and glutathione through cystathionine b-synthase (Kharbanda 2009). Aberrant methionine metabolism has been well documented in ALD (Kharbanda 2009, Halsted and Medici 2012, Kharbanda 2013). Ethanol exposure inhibits MS activity resulting in decreased hepatic methionine levels (Kharbanda 2009). Cells activate a compensatory pathway in methionine metabolism by increasing betaine homocysteine methyltransferase activity, but this pathway is compromised under extended chronic alcohol exposure resulting in a general decrease of hepatic SAM and essential methylation reactions (Kharbanda 2013).

Emerging evidence shows that post-translational modification of proteins with methylation may play important roles in regulating autophagy in at least three aspects: methylation of protein phosphatase 2A (PP2A) to negatively regulate autophagy through modulating mTOR (Sutter et al. 2013), epigenetic regulation of autophagy gene transcription through the methyltransferase G9a

(Artal-Martinez de Narvajas et al. 2013), and arginine methylation in selective autophagy (Li et al. 2013). In a recent yeast study, it was found that methionine and SAM inhibited autophagy and promoted growth through the protein phosphatase methyltransferase 1 (Ppm1p), which increases PP2A methylation. Methylated PP2A promoted the dephosphorylation of natriuretic peptide receptor B (Npr2), a yeast phosphoprotein that negatively regulates TORC1, resulting in TORC1 activation and autophagy inhibition (Sutter et al. 2013). However, whether methionine and SAM also inhibit autophagy in mammals through similar mechanisms remains to be studied. The H3K9 methyltransferase G9a was also reported to inhibit autophagy by inducing an increase of dimethylated H3K9 (H3Kme2), which repressed the expression of several essential Atg genes including LC3B, WIPI1 and diabetes-and obesity-regulated (DOR). Upon autophagy induction, G9a leaves the promoter region of *LC3B* to release its repression on the expression of *LC3B* and other Atg genes to promote autophagy (Artal-Martinez de Narvajas et al. 2013). For selective autophagy, it is known that the autophagy receptor complex is important for mediating recognition of cargos (such as ubiquitinated mitochondria) and also binds with autophagy machinery proteins (such as LC3), which allows the cargo to be selectively removed (Kirkin et al. 2009, Ding et al. 2010, Ding and Yin 2012, Manley et al. 2013). Phosphorylation of p62/SQSTM1 and Atg32, two important autophagy receptor proteins, has been shown to play important roles in selective removal of protein aggregates and mitochondria (Aoki et al. 2011, Matsumoto et al. 2011, Ichimura et al. 2013). Optineurin, another autophagy receptor protein, is also phosphorylated by the protein kinase TBK1, which enhances its binding with LC3 resulting in selective autophagic clearance of cytosolic *Salmonella enterica* (Wild et al. 2011). In addition to phosphorylation, arginine methylation is another major type of protein post-translational modification and is catalyzed by protein arginine methyltransferases (PRMT). Interestingly, a recent study showed that mutations in C. *elegans epg-11*, a homologue of mammalian *PRMT1*, led to the defective removal of P

granule components phenolic glycolipid-1 (PGL-1) and PGL-3. Furthermore, mutating the methylated arginine residues on PGL-1 and PGL-3 resulted in impaired degradation of PGL-1 and PGL-3 (Li et al. 2013). These results indicate that modification of autophagic cargo proteins by arginine methylation may provide a regulatory mechanism for modulating autophagic degradation efficiency during selective autophagy. As discussed above, alcohol consumption impairs methionine metabolism and methylation reactions. It will be interesting to determine how these methylation changes would affect selective autophagy (such as mitophagy and lipophagy) and general autophagy in alcohol-induced liver disease in the future. The possible autophagic signaling pathways or targets modulated by ethanol are summarized in Figure 1.4.1.

Figure 1.4.1

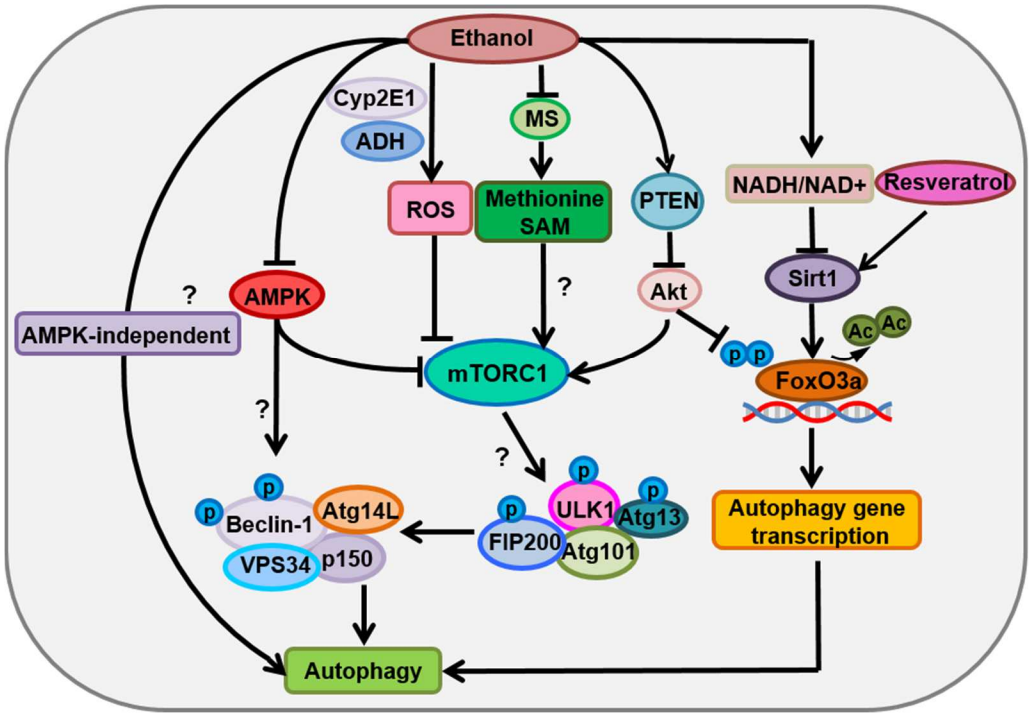


Figure 1.4.1 The major molecular pathways and targets in alcohol-induced autophagy changes in hepatocytes.

Ethanol modulates autophagy through multiple mechanisms: (1) Acute ethanol-induced autophagy requires ethanol metabolism and ROS production. ROS may activate autophagy by further suppressing mTOR. (2) Alcohol (ethanol) consumption inhibits methionine synthase (MS) resulting in decreased methionine and S-adenosylmethionine (SAM) levels. Methionine and SAM inhibit autophagy by activating mTORC1. Thus it is possible that ethanol-induced decreased methionine and SAM will inhibit mTORC1 resulting in autophagy activation although this has not been directly tested in the alcohol model (?). (3) Ethanol may also suppress Akt through the upregulation of PTEN and in turn inhibits mTORC1 to induce autophagy. (4) Ethanol-induced impaired AMPK and Akt may counteract each other on mTOR, and impaired Akt plays a dominant role toward the inhibition of mTOR. (5) Decreased Akt can also trigger autophagy through the activation of FoxO3 by promoting the dephosphorylation and nuclear retention of FoxO3. Increased NADH/NAD^+ ratio through ethanol metabolism inhibits Sirt1 activity resulting in increased acetylated FoxO3. Increased acetylated FoxO3 may decrease FoxO3-mediated expression of autophagy genes, which can be abolished by resveratrol that activates Sirt1. (6) Other AMPK-independent pathways remain to be determined in alcohol-induced autophagy (?). (7) mTORC1 negatively regulates autophagy through direct phosphorylation (p) of ULK1 to inactivate ULK1 complex activity. ULK1 directly phosphorylates Beclin-1 and enhances VPS34 kinase activity to promote autophagy. AMPK positively regulates autophagy by suppressing mTORC1 activity through phosphorylation of TSC2 and Raptor and by promoting VPS34 kinase activity through phosphorylation of Beclin-1. Activated VPS34 increases the production of phosphatidylinositol 3-phosphate, which promotes the biogenesis of autophagosomes although the activities of ULK1 and VPS34 after alcohol exposure still remain to be determined (?).

1.4.3 Autophagy and Effect of Alcohol on Adipose Tissue

Alcohol and Adipose Tissue Atrophy

Increasing evidence has shown that the crosstalk between adipose tissue and liver is important in liver diseases, since adipose tissue is not only a lipid storage organ, but also a crucial endocrine organ secreting various adipokines (Schaffler et al. 2005, Marra and Bertolani 2009). Adiponectin (Xu et al. 2003), an adipokine exclusively secreted by adipose tissue, and agonist of PPAR-γ that is a receptor crucial in maintaining adipose expansion and adiposity (Shen et al. 2010, Sun et al. 2012), could both improve lipid dysregulation and liver steatosis in mice.

As discussed above, hepatic steatosis is the hallmark of early ALD pathogenesis. Several mechanisms could contribute to alcohol-induced steatosis, including increased lipid uptake and synthesis, decreased FA β-oxidation and VLDL secretion, and changed levels of humoral factors (Donohue 2007, Williams et al. 2014). Epidemiology studies have shown that habitual moderate and heavy drinkers have less body mass index, especially in males (Liangpunsakul et al. 2010). It is generally agreed that alcohol exposure leads to adipose tissue atrophy. In contrast, aberrant distribution of body fat and excessive accumulation of adipose tissue may exacerbate the development of ALD, as abdominal height is positively associated with fibrosis score in ALD patients (Naveau et al. 2013). Abundant evidence in animal experiments also indicates that long-term alcohol intake induces adipose atrophy and disturbs lipid metabolism of adipose tissue, especially in WAT. Chronic alcohol feeding (4 weeks) in rats decreases white adipose tissue with increased TG degradation and increased lipolysis (Kang et al. 2007). Chronic alcohol exposure (8 weeks) in mice reduces WAT, stimulates adipose tissue lipolysis, and inhibits adipose FA uptake, while supplement of rosiglitazone, the PPARγ agonist, normalizes the adipose gene expression and corrected lipid dyshomeostasis (Sun et al. 2012, Zhong et al. 2012). In addition to increased

lipolysis in adipocytes, chronic feeding with ethanol in rats showed decreased expressions of lipogenic enzymes including PPAR-γ and C/EBPα in epididymal WAT in ethanol group, suggesting that adipocyte tissue atrophy may also due to decreased lipogenesis. Notably, visceral WAT with higher ALDH metabolism was more susceptible to ethanol treatment than subcutaneous WAT, and aldehyde treatment *in vitro* and *ex vivo* was sufficient to suppress lipogenic enzymes (Zhang et al. 2015). This suggests that aldehyde, a product of alcohol metabolism, inhibits lipogenesis and contributes to the lipodystrophy in adipose tissue.

Alcohol and Adipose Tissue Insulin Resistance, Inflammation and Lipolysis

Insulin is a hormone produced by pancreatic beta cells. It mediates metabolism of carbohydrates, lipids, and proteins in multiple organs (liver, adipose tissue, skeletal muscle, etc.) to uptake, store, and utilize the glucose from food intake. In adipose tissue, it mainly increases the rate of glucose transport and glycolysis, decreases the rate of lipolysis, stimulates FA and TG synthesis and uptake, and increases the rate of protein synthesis (Dimitriadis et al. 2011). Insulin resistance refers to the genetic or acquired condition in which the body does not respond well to insulin. As a result, the pancreas produces more insulin while the blood glucose remains elevated. While insulin resistance often happens in metabolic disorders like type II diabetes and obesity, binge drinking has also been shown to impair whole body insulin sensitivity. Oral or intraperitoneal administration of alcohol in rats for three consecutive days had worse insulin response, reflected by higher blood glucose level and lipolysis rate in adipose tissue (Lindtner et al. 2013). Chronic alcohol consumption also impairs the insulin sensitivity of adipose tissue. Isolated epididymal and subcutaneous adipocytes from rats fed ethanol for 4 weeks had impaired glucose uptake, and had decreased response to insulin's inhibition in β-adrenergic agonist-induced

glycerol release (Kang et al. 2007). Therefore, alcohol-induced adipocyte insulin resistance may also partially contribute to alcohol-induced adipose tissue atrophy by increasing lipolysis.

Insulin resistance and obesity is often associated with macrophage infiltration in adipose tissue. Insulin resistance and type II diabetes can often be associated with a switch from M2 (alternatively activated) macrophages secreting anti-inflammatory cytokines like Interlukin (IL)-10 to M1 (classically activated) macrophages secreting pro-inflammatory cytokines like TNF-α, IL-6, and IL1-β (Lumeng et al. 2007), and increased presence of crown-like structures, i.e. macrophages surrounding dead adipocytes (Suganami and Ogawa 2010). Notably, chronic ethanol feeding for 4 weeks in rats also resulted in increased macrophages infiltration into epididymal adipose tissue with elevated mRNA of TNF-α, IL-6, CCL2, and decreased mRNA of adiponectin and retinol binding protein 4 (RBP4) (Kang et al. 2007). Dr. Nagy's group has reported that chronic alcohol feeding (25 days) in mice induces apoptosis and inflammation in WAT in a CYP2E1/Bid/C1q-dependent manner (Sebastian et al. 2011). The same group also reported that chronic alcohol feeding (4 weeks) in rats suppresses adiponectin secretion and enhances markers of oxidative stress via CYP2E1 pathway (Tang et al. 2012). This suggests that ethanol metabolism and ethanol-induced adipocyte cell death promote inflammation in adipose tissue.

It is well known that elevated intracellular cAMP concentration and enhanced catecholamine signaling stimulate the β_2-adrenergic receptors on adipose tissue to increase lipolysis. Surprisingly, previous work from Dr. Nagy's group has reported that chronic ethanol feeding suppressed beta-adrenergic receptor-mediated lipolysis in adipocytes. They found that chronic ethanol feeding decreased beta-adrenergic receptor-stimulated protein kinase A activation that led to decreased phosphorylation of perilipin A and HSL. Moreover, chronic ethanol feeding increased phosphodiesterase 4 activity in adipocytes, resulting in decreased accumulation of cAMP (Kang

et al. 2007). In addition, epididymal adipocytes isolated from rats treated with chronic ethanol liquid diet exhibited similar basal lipolysis but decreased lipolysis in response to β-adrenergic agonist associated with increased phosphodiesterase 4 activity (Kang and Nagy 2006). However, chronic ethanol feeding markedly impaired insulin-mediated suppression of lipolysis in rats and in adipocytes isolated from epididymal and subcutaneous adipose tissue (Kang et al. 2007). These data suggest that alcohol induced adipose tissue atrophy is likely due to a suppression of the anti-lipolytic effects of insulin in adipocytes but may not directly affect beta-adrenergic receptor.

Notably, it is also reported that the change in lipoprotein lipase (LPL) activity, HSL activity and cAMP accumulation in WAT and BAT after ethanol exposure are very different depending on the dose and route of ethanol exposure, and tissue type (Shih and Taberner 1997). Moreover, a recent study also found that chronic feeding plus alcohol binge in mice increased adipose intracellular cAMP levels resulting in the activation of lipolytic enzymes and increased lipolysis, which can be reversed by supplying recombinant human fibroblast growth factor 21 (FGF21) (Zhao et al. 2015). Therefore it seems that the conditions of ethanol treatment may affect how ethanol induces adipocyte lipolysis. While the mechanisms for ethanol-induced adipocyte lipolysis are still controversial, it is consistent that alcohol consumption can lead to adipose tissue atrophy.

Alcohol and Adipose Tissue Protein Metabolism and Autophagy Activity

In addition to the known catecholamine and beta-adrenergic receptor-mediated lipolysis and insulin-mediated anti-lipolysis signaling in alcohol-induced adipocyte lipolysis, several other mechanisms are emerging that may also contribute to alcohol-induced adipose tissue homeostasis and lipolysis.

Chronic alcohol consumption is known to cause mass loss and dysregulated protein metabolism in skeletal muscle (Steiner and Lang 2015). Investigators found that the mechanisms include inhibited global protein synthesis (Preedy et al. 1997) and increased autophagy degradation (Thapaliya et al. 2014). Surprisingly, a recent study showed that mice with chronic ethanol feeding for 24 weeks had increased protein synthesis, which was associated with increased phosphorylation of mTOR substrate proteins and increased expression of Atg genes (Crowell et al. 2016). It is likely that increased mTOR-mediated protein synthesis may reflect an adaptive response of adipose tissue in response to alcohol-induced adipose tissue atrophy. While the authors found increased expression of Atg genes after alcohol exposure, no autophagy flux assay was performed in this study. It thus remains unclear whether autophagy is increased in adipocytes in this model. As discussed above, adipocyte-specific *Atg5* or *Atg7* KO mice have decreased adipose tissue mass, which is similar to chronic alcohol-induced adipose tissue atrophy. One would assume that chronic alcohol may also impair autophagy in adipocytes. To determine autophagic flux *in vivo* such as mouse adipose tissue is technically challenging, better animal models and autophagic flux markers need to be developed to tackle this important question in the future.

1.4.4 Fat-liver Axis in Alcoholic Liver Disease

Adipose tissue is an endocrine organ and secretes many bioactive substances, collectively called adipokines. So far, evidence suggests that more than 600 adipokines play specific roles in immune response, inflammation, glucose/lipid metabolism, insulin sensitivity, regulation of appetite and satiety, adipogenesis and bone morphogenesis, and other biological processes (Kloting and Bluher 2014). Excess adiposity or adipocyte dysfunction leads to dysregulation of

adipokines and nutritional metabolites, and thus affects other organs like liver (Girard and Lafontan 2008, Reilly et al. 2015) and skeletal muscle (Argiles et al. 2005). Correspondingly, the disorder in liver and skeletal muscle (Kim et al. 2013) can also affect the mass and functioning of adipose tissue. Below we discuss potential pathways of the crosstalk between adipose tissue and liver.

Adiponectin

Adiponectin or 30 kDa adipocyte complement-related protein (Acrp30) encoded by *Adipoq* is a protein hormone predominantly expressed in adipose tissue. Serving as an anti-diabetic adipocytokine, it regulates glucose level and FA breakdown. Metabolically it reduces body fat and improves insulin sensitivity (Berg et al. 2001). Moreover, adiponectin has anti-inflammatory effects such as blocking macrophage function (Yokota et al. 2000) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling (Ouchi et al. 2000). Different forms of adiponectin supplementation have shown the potency of treating obesity, diabetes and insulin resistance (Haluzik 2005). Adiponectin has two receptors adiponectin receptor 1 (AdipoR1) and AdipoR2, and the latter is highly expressed in liver. Adiponectin is able to regulate liver steatosis, insulin resistance, inflammation and fibrosis by binding to these two receptors (Moschen et al. 2012), all of these are important for the pathogenesis of ALD. Indeed, chronic alcohol feeding decreases circulating adiponectin in mice and rats (Chen et al. 2007), and supplementation of recombinant adiponectin alleviates alcohol-induced liver steatosis and injury (Xu et al. 2003). These data clearly support the notion that modulating adipose tissue function can affect the pathogenesis of ALD.

FGF21

FGF21 is a member of FGF family. It is preferentially expressed in liver, and is inducible in adipose tissue (Owen et al. 2015). As an insulin-sensitizing metabolic regulator, it stimulates glucose uptake on adipocytes and also targets central nervous system (Markan et al. 2014). FGF21 treatment corrects diet-induced obesity in mice, which suggests its therapeutic potency in obesity and fatty liver (Coskun et al. 2008). Moreover, *Fgf21* transgenic mice are resistant to diet-induced obesity (Kharitonenkov et al. 2005).

Increasing evidence indicates that FGF21 also regulates the pathogenesis of ALD at multiple levels. *Fgf21* KO mice have increased sucrose consumption, whereas acute administration or overexpression of FGF21 suppresses the intake of both sugar and non-caloric sweeteners. FGF21 regulates the neuron functions in the hypothalamus as a negative feedback loop in the liver-to-brain hormonal axis, which reduces sweet-seeking behavior and meal size. Thus FGF21 acts centrally to suppress the intake of "sweets" by producing an endocrine satiety signal (von Holstein-Rathlou et al. 2016). A recent study also showed that FGF21 administration reduces sweet and alcohol preference in mice and sweet preference in cynomolgus monkeys. In mice, the reduced preferences for sugar and alcohol require the FGF21 co-receptor β-Klotho in the central nervous system, which are also correlated with reductions in dopamine concentrations in the nucleus accumbens (Talukdar et al. 2016). A recent genome-wide association meta-analysis and replication study among a large population of individuals of European ancestry has identified β-Klotho as a locus associated with alcohol consumption. In addition, brain-specific *Klb* KO mice increase alcohol preference, whereas FGF21 administration inhibits alcohol drinking by acting on the brain (Schumann et al. 2016). Therefore, in addition to regulating uptake of sweetness, the liver-brain endocrine axis may also play a critical role in the regulation of alcohol drinking

behavior. Since analogs of FGF21 are currently undergoing clinical evaluation for the treatment of obesity and type II diabetes, FGF21 may also be a potential pharmacologic target for reducing alcohol consumption.

In addition, both hepatic and serum FGF21 levels increased after mice were either challenged with acute binge, chronic-plus-binge alcohol, or alcohol chronic feeding for 4 weeks. However, different alcohol models led to different outcomes for liver injury in *Fgf21* KO mice (Zhao et al. 2015, Liu et al. 2016, Desai et al. 2017). Chronic-plus-binge alcohol-induced liver injury and steatosis were attenuated in *Fgf21* KO mice. In contrast, *Fgf21* KO mice had more severe steatosis and liver injury compared with wild type mice when they were fed with chronic alcohol for 4 weeks. The reasons for these apparent contradictory results were not clear but it suggests that different alcohol exposure model may be responsible for these contradictory findings. Notably, it is known that chronic-plus-binge model often leads to more hepatic neutrophil infiltration and inflammation, which are absent in the chronic alcohol feeding model. Whether lack of FGF21 would differentially affect liver inflammation need to be further studied.

As discussed above, chronic alcohol consumption reduces adipose tissue mass by increasing lipolysis. Interestingly, FGF21 deficiency also markedly reduces alcohol-induced WAT lipolysis, and administration of exogenous FGF21 exacerbates chronic alcohol-induced lipolysis. Mechanistically, the authors did not find any changes in the insulin levels but the levels of catecholamine decreased in *Fgf21* KO mice (Zhao et al. 2015). Regardless of these controversial findings in chronic alcohol or chronic-plus-binge alcohol-treated *Fgf21* KO mice, recombinant FGF21 treatment ameliorates liver injury and steatosis induced by alcohol (Zhu et al. 2014, Liu et al. 2016). Whether FGF21 also affects autophagy in adipose tissue and in turn affects the

pathogenesis of ALD remains to be an unanswered interesting question, which is currently under active investigation in our lab.

1.4.5 Autophagy in Other Tissue Injuries Induced by Alcohol

Heart

Heart is mainly comprised of long-lived and post-mitotic cardiomyocytes. Increasing evidence indicates that autophagy plays an important role in maintaining the function and viability of cardiomyocytes by controlling the homeostasis of intracellular proteins, energy and organelles (Lavandero et al. 2013). Studies from genetic KO animal models, such as using the cardiomyocyte-specific *Atg5* KO mice, revealed that basal autophagy plays a vital housekeeping role in removing damaged organelles and proteins in cardiomyocytes to maintain their normal functions (Nakai et al. 2007). In contrast, both protective and detrimental roles of autophagy have been reported in "stressed" or "diseased" heart. For example, induction of autophagy is protective against ischemia-induced heart injury, whereas autophagy could be detrimental in pressure overload-induced heart failure and during reperfusion (Matsui et al. 2007, Zhu et al. 2007).

Low to moderate alcohol consumption is beneficial to patients with cardiovascular events (Costanzo et al. 2010). In contrast, heavy alcohol consumption impairs cardiac geometry and function (Ren and Wold 2008), and increases the incidence of sudden cardiac death and ventricular arrhythmias (Spies et al. 2001). Whether autophagy plays a protective or detrimental role in alcoholic heart disease is not fully understood. Jun Ren's team has conducted a series of studies on ethanol-induced cardiac dysfunction with a focus on autophagy (Ge et al. 2011, Ge and Ren

2012, Guo et al. 2012, Guo and Ren 2012, Kandadi et al. 2013). In both binge (Ge et al. 2011) and chronic (Ge and Ren 2012) alcohol models, heart LC3-II levels were increased in an AMPK-dependent manner. Furthermore results from this group's studies tend to suggest that autophagy may contribute to alcohol-induced malfunction of cardiomyocytes. Acute ethanol treatment led to compromised heart functions with decreased fractional shortening, peak shortening and an intracellular Ca^{2+} rise in mouse cardiomyocytes. Acute ethanol exposure also increased LC3-II level, which was accompanied by increased phosphorylation of AMPK, Raptor, and decreased phosphorylation of mTOR and ULK1 in mouse cardiomyocytes. Interestingly, pharmacological or genetic inhibition of AMPK attenuated ethanol-induced autophagosome formation and cardiomyocyte apoptosis. Moreover, 3-methyladenine (3-MA) reversed ethanol-induced cardiomyocyte contractile defects (Guo and Ren 2012). Similar to acute ethanol treatment, chronic ethanol feeding also led to increased autophagosome formation in mouse cardiomyocytes with heart hypertrophy and cardiomyocyte contractile anomalies, and 3-MA treatment also ablated these ethanol-induced cardiomyocyte malfunction (Guo et al. 2012). Moreover, mice with cardiac-specific overexpression of ADH, which metabolizes alcohol to acetaldehyde, were more susceptible to ethanol-induced autophagy changes and ethanol-induced damage of cardiomyocytes (Guo et al. 2012). In contrast, transgenic mice overexpressing ALDH2, which converts acetaldehyde to acetate during alcohol metabolism, blunted chronic alcohol-induced mTOR inhibition and increased LC3-II levels resulting in improved cardiac geometry and function in alcohol-treated mice (Ge and Ren 2012). These findings suggest that the ethanol metabolite acetaldehyde may account for ethanol-mediated autophagy changes and impaired cardiac functions.

While these data generally suggest that either acute or chronic ethanol treatment may induce autophagy and contribute to ethanol-induced malfunction of cardiomyocytes, no clear autophagic flux data were shown in these studies. It is intriguing that acute ethanol treatment increased both LC3-II and p62/SQSTM1 in mouse cardiomyocytes (Guo and Ren 2012), although it is not clear whether the increased p62/SQSTM1 was due to decreased degradation or increased transcription. More studies, in particular the use of genetic autophagy-deficient animal models, are definitely needed to further clarify the autophagy status after alcohol exposure and the exact role of autophagy in alcohol-induced heart dysfunction.

Brain

It is well established that excessive ethanol intake results in regional brain damage and cognitive dysfunction (Harper and Matsumoto 2005, Harper 2009). Potential mechanisms that are responsible for alcohol-induced brain injury include higher sensitivity for excitotoxicity, impaired catabolism of homocysteine, reduced neurotrophic factors, failure to repair damaged DNA, acetaldehyde adduct formation and so on (Harper and Matsumoto 2005). It is generally thought that in the mature mammalian brain, autophagy is hard to detect even under nutrient deprivation conditions (Mizushima et al. 2004). This is probably due to the vital functions of brain that need to be protected from even systemic nutrient deprivation. However, neural cell-specific *Atg5* KO mice have increased accumulation of cytoplasmic inclusion bodies in neurons and develop progressive deficits in motor function, suggesting that basal autophagy in the brain is important for preventing the accumulation of abnormal proteins to preserve neural function and protects against neurodegeneration (Hara et al. 2006). Moreover, both increased and impaired autophagy have also been observed in various acute brain injuries including those induced by alcohol (Smith et al. 2011, Chen et al. 2012).

Ethanol treatment increases autophagic flux in SH-SY5Y neuroblastoma cells and in the developing mouse brain through inhibition of mTOR. More importantly, induction of autophagy by rapamycin attenuates ethanol-induced ROS production and neuronal cell death in SH-SY5Y cells and in the mouse developing brain. In contrast, inhibition of autophagy either by wortmannin or shRNA knockdown of Beclin 1 exacerbates ethanol-induced neurotoxicity (Chen et al. 2012). Moreover, hypoxic preconditioning activates autophagy and protectes against ethanol-induced neurotoxicity, which is abolished when autophagy is inhibited by either BAF or wortmannin (Wang et al. 2013). These results suggest that autophagy protects against ethanol-induced neuronal injury. Fetal alcohol spectrum disorder (FASD) results from prenatal exposure to alcohol, which is the leading cause of mental retardation. Children with FASD often have neuropsychological and behavioral problems, and develop secondary disabilities including depression and anxiety disorder. Alimov *et al*. (Alimov et al. 2013) found that subcutaneous injection with ethanol induced neuroapoptosis in postnatal day 4 mice but not in postnatal day 12 mice. Interestingly, they further found that the expression of genes that regulate autophagy and the UPR was lower whereas the expression of pro-apoptotic genes was higher in postnatal day 4 mice than postnatal day 12 mice. These results imply that decreased autophagy activity may contribute to the vulnerability of the immature brain to alcohol exposure. However, a more recent study found that mice administrated 10% (v/v) ethanol for 4 months led to an accumulation of poly-ubiquitinated proteins in the mouse cerebral cortex likely due to an impaired ubiquitin-proteasome system and autophagy. Specifically, it was found that ethanol treatment increased mTOR activity and decreased expression of several Atg genes including *Atg12*, *Atg5*, *p62/SQSTM1* and *LC3*. Ethanol treatment also increased brain inflammatory mediators such as IFN-γ. Interestingly, these ethanol-induced changes were attenuated in Toll-like receptor 4 (*Tlr4*) KO mice, which were protected against chronic ethanol exposure-induced brain injury (Pla et al. 2014). These results suggest that

ethanol-induced impairment of the ubiquitin proteasome system and autophagy could be due to the activation of TLR4 by inflammatory mediators. In the future, more studies are needed to determine whether autophagy is activated or impaired after alcohol consumption using different animal models such as chronic-plus-binge model. Moreover, it will also be interesting to determine whether the metabolism of ethanol is required for ethanol-induced changes on autophagy in brain. Nevertheless, it seems that activation of autophagy is beneficial for alcohol-induced brain injury.

Skeletal Muscle

Skeletal muscles are composed of myofibers that control our body's motion. Myofibers are composed of myofibrils that form highly organized units called sarcomeres, which contain repeating actin and myosin proteins. Approximately 40% of our body mass is from skeletal muscle, which also plays a critical role in regulating metabolism by providing amino acids through breaking down proteins and organelles to meet the energy needs of the body (Sandri 2010). Thus, it is not surprising that emerging evidence suggests that autophagy is important for controlling muscle mass. Modulating muscle autophagy also influences exercise, energy and lipid metabolism (Sandri 2010, Neel et al. 2013). Both beneficial and deleterious roles of autophagy in regulating muscle mass/wasting have been proposed. Activation of FoxO3 led to increased expression of Atg genes and activation of autophagy, which resulted in muscle atrophy (Mammucari et al. 2007, Zhao et al. 2007). MTMR14 is a lipid phosphatase that antagonizes Vps34 to dephosphorylate PI3-P to phosphatidylinositol (PI) and thus inhibits autophagy. Increased autophagy and muscle atrophy have been reported in *MTMR14* knockdown zebrafish (Dowling JJ 2010; Vergne I 2009). Moreover, centronuclear myopathy was also found in humans that have *MTMR14* mutations (Tosch et al. 2006). Paradoxically, muscle-specific *Atg7* KO mice also developed myofiber degeneration and muscle atrophy accompanied with increased accumulation of protein aggregates,

abnormal mitochondria, sarcoplasmic reticulum distension, vacuolization, increased oxidative stress and apoptosis (Masiero and Sandri 2010). It is possible that autophagic degradation of proteins may lead to muscle atrophy whereas the muscle atrophy observed in the muscle autophagy-deficient mice is a maladaptive response due to the chronic loss of autophagy.

In addition to regulating muscle mass, autophagy in muscle also regulates body glucose and lipid metabolism. For example, exercise induces autophagy in multiple organs involved in metabolic regulation including muscle, liver, pancreas and adipose tissue (He et al. 2012). Exercise increases Bcl-2 phosphorylation resulting in its dissociation from Beclin-1, which leads to the initiation of autophagy. Nonphosphorylatable mutation in Bcl-2 (Thr69Ala, Ser70Ala and Ser84Ala, Bcl2 AAA) knockin mice cause them to be defective in exercise- and starvation-induced autophagy, and they show decreased exercise endurance. These defects are due to impaired exercise-induced skeletal muscle glucose uptake because of a loss in glucose transporter 4 (GLUT4) translocation (He et al. 2012, He et al. 2012). These findings suggest that autophagy may be beneficial for glucose homeostasis during exercise. Moreover, studies from muscle-specific *Atg7* KO mice also reveal that autophagy in muscle may regulate glucose and lipid homeostasis (Kim et al. 2013). *Atg7* muscle-specific KO mice have decreased fat mass and are resistant to high fat diet-induced obesity and insulin resistance. Mechanistically, it has been suggested that loss of *Atg7* may lead to accumulation of damaged mitochondria, which induces an Atf4-dependent production of FGF21 that increases FA oxidation and browning of WAT (Kim et al. 2013). Thus, these seemingly beneficial effects of loss of muscle autophagy on glucose and lipid metabolism could be a secondary adaptive response in response to organelle damage induced by the loss of autophagy. It is not clear how long these adaptive responses would last and whether maladaptive responses would develop after long term loss of muscle autophagy.

It is well known that chronic alcoholics have severe muscle loss and myopathy. Both *in vivo* and *in vitro* studies show that ethanol can inhibit skeletal muscle protein synthesis, which is likely mediated by increased expression of insulin-like growth factor binding protein-1 and myostatin [a transforming growth factor beta (TGF-β) superfamily member] resulting in the inhibition of mTOR and limitation of translational efficiency (Preedy et al. 1997, Lang et al. 1998, Lang et al. 2004). Using skeletal muscle biopsies from alcoholic cirrhotics, gastrocnemius from ethanol and pair-fed mice, and ethanol-exposed murine myotubes, Thapaliya et al. provided evidence that autophagy contributes to alcohol-induced skeletal muscle loss (Thapaliya et al. 2014). Using a standard CT imaging technique, it was found that alcoholic cirrhotics had lower muscle mass than controls. Interestingly, proteasome components and activity were decreased in alcoholic biopsy samples, suggesting that decreased skeletal mass in alcoholic cirrhotics is less likely mediated by the proteasome. Indeed, they found that the expression of several essential Atg genes and autophagic flux were increased in alcoholic biopsy samples, ethanol-fed mice and ethanol treated C2C12 murine myotubes. Alcohol-induced autophagy was mediated by acetaldehyde, the metabolite of ethanol, rather than ethanol *per se.* More importantly, pharmacological or genetic inhibition of autophagy mitigated the proteolysis of myotubes and the reduction of muscle mass (Thapaliya et al. 2014). However, most of the results were obtained from short term alcohol exposure experiments. It is uncertain whether long-term blockage of autophagy would be beneficial for alcohol-induced muscle loss. More studies are needed to further dissect the underlying mechanisms by which autophagy regulates skeletal muscle mass in alcoholics.

Pancreas

Pancreas is a glandular organ that has both endocrine and exocrine functions in vertebrates. In response to fasting or feeding, pancreas secretes insulin or glucagon through its endocrine

system (islet β or α cells) to maintain blood glucose levels. Acute and chronic pancreatitis are characterized by intracellular activation of digestive enzymes, intracellular vacuolization, apoptosis, necrosis, edema and inflammation (Gaisano and Gorelick 2009, Gukovskaya and Gukovsky 2012). Chronic pancreatitis is also associated with a high risk for the development of pancreatic adenocarcinoma, for which no treatment is currently available (Chen and Ferec 2009, Gukovsky et al. 2013).

Similar to the animal models for alcohol-induced liver injury, neither acute nor chronic administration of ethanol alone in rodents leads to pancreatitis. Alcohol-induced pancreatitis requires other additional factors such as a viral infection, a high fat diet or submaximal postprandial dose of CCK or cerulein or cholinergic stimulation (such as by carbachol). The precise mechanisms by which alcohol induces pancreatic damage remain vague. A series of elegant published work from Gaisano's group showed that alcohol consumption may alter apical and basolateral exocytosis in pancreatic acinar cells (Cosen-Binker et al. 2007, Cosen-Binker et al. 2008). Mechanistically, it has been shown that alcohol induced protein kinase Cα, which phosphorylated Munc18c and displaced it from binding to basolateral plasma membrane syntaxin 4 (Syn-4), which results in formation of the Syn4/ synaptosomal-associated protein 23 (SNAP23)/ vesicle-associated membrane protein 8 (VAMP8) fusion complex. The Syn4/SNAP23/VAMP8 fusion complex then redirected the zymogen from apical exocytosis to basolateral exocytosis, which causes pancreatitis (Cosen-Binker et al. 2007, Lam et al. 2007, Cosen-Binker et al. 2008). Chronic ethanol feeding promotes a shift of acinar cell apoptosis to necrosis, but little is known about the mechanisms involved. In pancreas, ethanol is metabolized through both oxidative and non-oxidative pathways. Oxidative metabolism of ethanol is mediated by ADH in cytosol and ALDH2 in mitochondria, which generates acetaldehyde and acetate, respectively. Non-oxidative

metabolism converts ethanol to FAEE via fatty acyl responsive regulator (FarR) synthase. It has been shown that oxidative metabolism of ethanol causes mitochondrial failure by activating mitochondrial permeability transition, a key event in regulating cell death (Shalbueva et al. 2013). However, whether ethanol feeding would affect other necrotic proteins such as RIP3 in pancreas is not known although RIP3 is important in mediating ethanol feeding-induced liver injury in mice (Roychowdhury et al. 2013). In addition, as discussed above, ethanol feeding may also induce ER stress in acinar cells to trigger cell death (Lugea et al. 2011). Alcohol consumption can increase gut permeability, which causes bacterial translocation across the mucosal barrier, and leads to the elevation of lipopolysaccharide (LPS) levels. Alcohol-fed rats that were further treated with LPS had increased expression of TGF-β, which led to subsequent pancreatic fibrosis (Gu et al. 2013).

Increasing evidence now supports the role of autophagy in both alcoholic and non-alcoholic pancreatitis, and it is generally thought that lysosomal/autophagic dysfunction can initiate pancreatitis. It has long been noted that there is an increased accumulation of large intracellular vacuoles in acinar cells in both experimental and human pancreatitis, and recent evidence indicates that the nature of these vacuoles is autophagic and lysosomal origin because these structures have double-membrane and are positive for LC3-II. In cerulein-induced acute alcoholic pancreatitis (AP), there was an increase in autophagosome numbers but autophagic flux was impaired due to lysosomal dysfunction (Gukovsky and Gukovskaya 2010). Decreased LAMP2 proteins and possible fusion of autophagosomes with lysosomes were also found in alcohol and LPS-induced AP (Fortunato et al. 2009). Moreover, *Xbp1*+/- mice fed with chronic ethanol had increased acinar cell death with loss of enzymogen granules. These mice also had increased LC3-II levels in acinar cells although autophagic flux assay was not performed in this study (Lugea et al. 2011). Mechanistically, autophagy may help remove zymogens through a selective process termed

zymophagy, which is regulated by the vacuole membrane protein (VMP1)-USP9x-p62/SQSTM1 complex and attenuates intra-acinar trypsinogen activation. VMP1 interacts with Beclin-1 to promote the formation of autophagosomes, and it also interacts with the ubiquitin-protease USP9x to induce selective zymophagy, which prevents acinar cell death (Ropolo et al. 2007, Grasso et al. 2011). In addition, results from Gukovskaya's group suggest that inefficient autophagic degradation of zymogens due to defective lysosomal proteolytic activity may promote pancreatitis. They also proposed that an imbalance between cathepsin B (CatB) and cathepsin L (CatL) may result in decreased degradation of trypsin, which leads to pancreatitis (Mareninova et al. 2009, Gukovskaya and Gukovsky 2012). Moreover, mice with pancreas specific deletion of IKK-α, an essential component for NF-κB activation, develop spontaneous pancreatitis. Interestingly, decreased autophagic flux is found in the mouse pancreas with specific deletion of IKK-α. Similar to the autophagy-deficient liver, increased p62/SQSTM1 levels were also found in the IKK-α-deficient pancreas, and further deletion of p62/SQSTM1 in the pancreas attenuated pancreatitis in pancreas-specific IKK-α-deficient mice (Li et al. 2013). Taken together, it seems that all of the above evidence supports that impaired autophagy may contribute to pancreatitis. Nevertheless, an early study using pancreas acinar cell-specific *Atg5* KO mice showed decreased acinar cell vacuolization and pancreatitis after cerulein treatment, and the authors proposed that autophagy machinery may be required for the trypsinogen activation to induce pancreatitis (Hashimoto et al. 2008). These results from acinar cell-specific *Atg5* KO mice seem to be contradictory to the above other findings that suggest impaired autophagy promotes pancreatitis. However, Atg5 mainly regulates the upstream formation of autophagosomes, and it is possible that upstream autophagy (autophagosome biogenesis) and downstream of autophagy (autolysosome degradation) could play different roles in pancreatitis. Trypsinogen may use autophagosomes as vehicles for transport to lysosomes where trypsinogen is activated. Indeed, inhibition of the early phase of autophagy by

3-MA completely blocked trypsinogen activation (Gukovsky and Gukovskaya 2010). In contrast, impaired functions of downstream autolysosomes also led to trypsinogen activation and pancreatitis (Gukovskaya and Gukovsky 2012, Gukovsky et al. 2012). Therefore, it is possible that targeting different phases of autophagy may lead to different outcomes of pancreatitis. Suppression of early phase autophagosome formation and improvement of late autolysosome functions may attenuate pancreatitis, but future experiments are needed to test this hypothesis.

1.4.6 Summary

Recent rapid research progress has significantly enriched our knowledge on the molecular mechanisms regulating autophagy and its impact on human diseases. As outlined in this section, autophagy plays significant roles in alcohol consumption-induced multiple tissue/organ injuries including hepatic steatosis and liver injury, impaired heart function, brain damage, loss of muscle mass, and pancreatitis,. While autophagy has been generally considered as a cell survival mechanism, both beneficial and detrimental effects of autophagy have been reported in alcohol-induced multiple tissue/organ injuries (Table 5). As a critical cellular mechanism sentinel for the homeostasis of proteins, energy, and organelles, autophagy may be beneficial for alcohol-induced liver injury through removing damaged mitochondria and lipid droplets, for brain injury through inhibiting ROS generation, and for AP through preventing zymogen activation. However, autophagy seems to be detrimental for alcohol-induced heart malfunction and muscle atrophy, although more studies are needed to further confirm these concepts due to limited research and lack of clear autophagic flux data in these two areas (Fig.1.4.2). Given the dynamic nature of autophagy and the chronic alcohol consumption process, we are still facing great challenges to monitor the autophagy status *in vivo* for chronic diseases induced by alcohol consumption. Similarly, it is also difficult to monitor the autophagy status *in vivo* after chronic modulation of

autophagy using either pharmacological autophagy inducers or inhibitors. More reliable *in vivo* autophagic flux assays are urgently needed to help further assess the therapeutic potential of pharmacological modulation of autophagy as a means to treat alcohol-induced tissue injuries.

Table 5 Summary of *in vivo* studies on autophagy in alcohol-induced tissue injury

Organ	Model	Level of autophagy	Role of autophagy	References
Liver	Acute	Activated	Protective	(Ding et al. 2010, Ni et al. 2013, Thomes et al. 2013)
		Impaired(*)	Protective	(Wu et al. 2012, Yang et al. 2014) (Zeng et al. 2012)
	Chronic	Impaired	Protective	(Donohue 2009, Lin et al. 2013, Chao et al. 2018)
Heart	Acute	Activated (*)	Detrimental	(Ge et al. 2011, Guo and Ren 2012, Kandadi et al. 2013)
	Chronic	Activated (*)	Detrimental	(Ge and Ren 2012, Guo et al. 2012)
Brain	Acute	Activated	Protective	(Chen et al. 2012, Alimov et al. 2013, Wang et al. 2013)
	Chronic	Impaired	Protective	(Pla et al. 2014)
Skeletal Muscle	Chronic	Activated	Detrimental	(Thapaliya et al. 2014)
Pancreas	Chronic	Impaired	Protective	(Gukovsky et al. 2008, Fortunato et al. 2009)

Note: * Autophagy flux assay is lacking

Figure 1.4.2

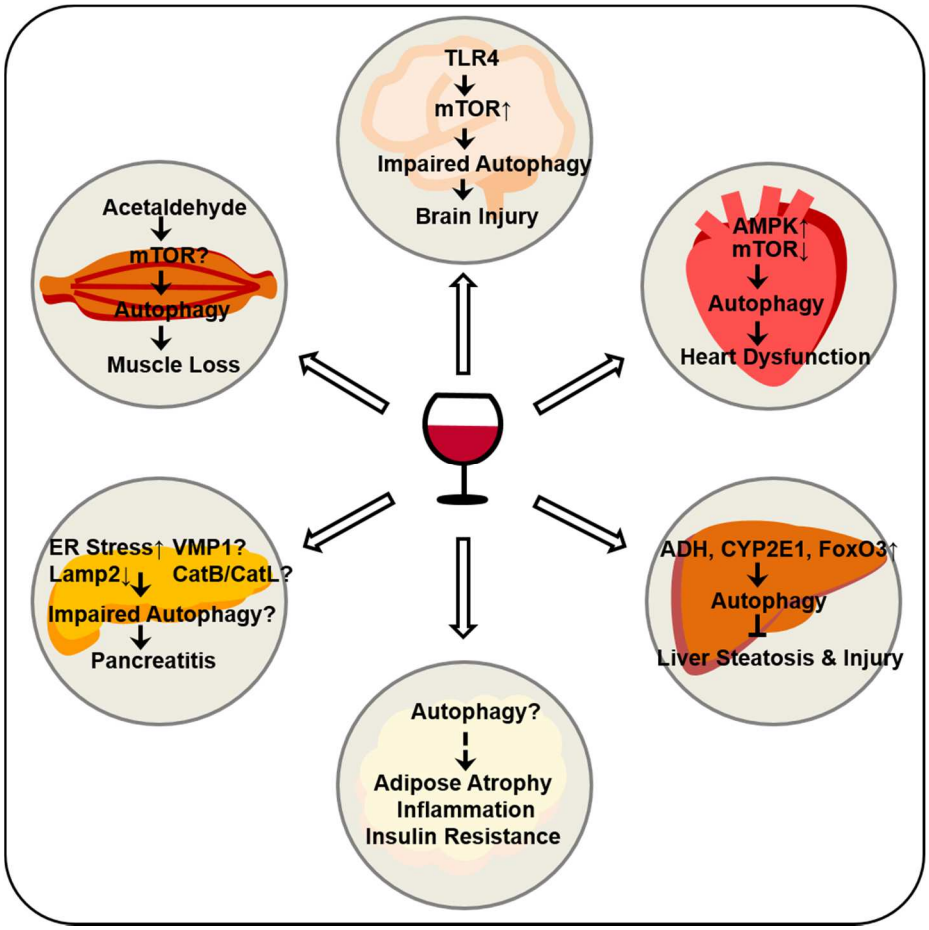


Figure 1.4.2 Differential roles of autophagy in alcohol-induced multi-tissue injury.

Emerging evidence now indicates that alcohol consumption can either activate or impair autophagy as either a cellular adaptive/compensatory protective mechanism or as a detrimental factor contributing to alcohol-induced injury in various tissues/organs. In liver, it seems that alcohol metabolism through ADH and CYP2E1 is required for autophagy activation. Acute alcohol treatment also induces FoxO3-mediated autophagy. Autophagy seems to selectively remove damaged mitochondria and excess lipid droplets and in turn attenuate alcohol-induced steatosis and liver injury. In heart, alcohol may activate autophagy through activating AMPK and inactivating mTOR. Autophagy activation seems to contribute to alcohol-induced heart dysfunction. In muscle, metabolism of alcohol to acetaldehyde activates autophagy resulting in muscle loss. Whether alcohol-induced autophagy in muscle is mediated by mTOR is not clear. In brain, alcohol increases mTOR and impairs autophagy in the mouse cerebral cortex resulting in brain injury, which is TLR4-dependent. In pancreas, alcohol can induce ER stress and also decrease LAMP2 in the presence of endotoxin LPS, which leads to impaired autophagy resulting in pancreatitis. It is not known whether alcohol consumption would affect VMP1 and the ratio of CatB (cathepsin B) /CatL (cathepsin L), two important factors that regulate autophagy and pancreatitis, respectively. In adipose tissue, whether autophagy is involved in alcohol-induced atrophy, inflammation and insulin resistance is not known and will be determined by this study.

1.5 Specific Aims

1.5.1 Specific Aim 1: Investigate the phenotype of A-Atg5 KO mice under chronic-plus-binge alcohol treatment

Autophagy is essential in adipose tissue development and physiological functions. We previously observed that chronic-plus-binge alcohol treatment and 32-day chronic alcohol treatment led to smaller adipose tissues along with mild liver injury and steatosis. I hypothesized that inhibiting adipose autophagy by specific *Atg5* deletion would lead to adipose tissue dystrophy and dysfunction, and subsequently enable mice to be more resistant to alcohol-induced adipose atrophy and ALD. My goal in this aim was to characterize the phenotype of A-Atg5 KO mice and their response to chronic-plus-binge alcohol, especially the pathogenesis induced by alcohol in adipose tissue and liver.

1.5.2 Specific Aim 2: Investigate the role of alcohol on adipose autophagy and the crosstalk between adipose tissue and liver in ALD

As discussed above, mice with autophagy deficiency in adipose tissue have decreased adipose mass. I hypothesized that alcohol would enhance adipose autophagy, and mice with chronic autophagy deficiency in adipose tissue would be more resistant to ALD. A possible mechanism is that chronic autophagy deficiency decreases adipose tissue mass (increased lipolysis and/or decreased lipogenesis) resulting in less FA uptake by the liver after alcohol treatment. My goal in this aim is to determine the effect of chronic-plus-binge alcohol on adipose autophagy, and if autophagy and/or other mechanisms contribute to ALD.

1.5.3 Specific Aim 3: Investigate the effect of alcohol and autophagy in preadipocyte differentiation

Modulation of autophagy affects adipose tissue differentiation *in vivo* and *in vitro*, but the underlying mechanism is not fully understood. 3T3-L1 cells can be differentiated into adipocyte-like cells under proper pharmacological induction. I hypothesized that alcohol would impair 3T3-L1 preadipocyte differentiation partly via affecting autophagy. I aimed to utilize 3T3-L1 cell line and other *in vitro* models to determine the effect of alcohol on preadipocyte adipogenesis and adipocyte autophagy.

CHAPTER 2. ADIPOSE AUTOPHAGY IN ALCOHOL-INDUCED ADIPOSE ATROPHY AND LIVER DISEASE

2.1 Abstract

Autophagy is a highly conserved lysosomal degradative pathway essential in maintaining cellular homeostasis. Mice with autophagy-deficiency in adipose tissue presented impaired adipogenesis and altered metabolic parameters. Accumulating evidence indicates that chronic alcohol consumption also induces adipose tissue atrophy in both mice and human. However, whether alcohol-induced adipose atrophy is through autophagy, and more importantly whether autophagy-mediated adipose atrophy would contribute to alcohol-induced liver injury is unclear. In this study, we generated Adipose-specific Atg5 (A-Atg5) KO mice using *Adipoq Cre* and challenged these mice with chronic-plus-binge alcohol exposure. Mice given calorie-matched feeding and binge were used as controls, and A-Atg5 KO mice and matched Cre-negative (Cre-) littermates were compared. We found chronic-plus-binge alcohol inhibited Akt/mTOR signaling and enhanced autophagy degradation in epididymal adipose tissue. In addition, chronic-plus-binge alcohol decreased adipocyte/LD size in WT mice, but A-Atg5 KO mice were resistant to alcohol-induced adipose atrophy and demonstrated increased browning in subcutaneous adipose tissue. Moreover, A-Atg5 KO mice were protected from alcohol-induced liver injury but not steatosis. Interestingly, A-Atg5 KO mice had higher circulating adiponectin at baseline, which may prime A-Atg5 KO mice to be resistant to alcohol-induced liver injury. In conclusion, constitutional autophagy deficiency in adipose tissue leads to reduced sensitivity to alcohol-induced adipose atrophy, and ameliorated alcohol-induced liver injury.

2.2 Introduction

Alcohol-induced liver disease (ALD) is a severe health issue in U.S. and worldwide. Currently no successful treatments are available for ALD. As mentioned in chapter 1.2, autophagy is crucial in adipose tissue development and function. To specifically study role of autophagy in adipose tissue, I used *Adipoq-Cre* to delete *Atg5* in mouse adipose tissue by crossing *Adipoq-Cre* mice with $Atg5^{flox/flox}$ ($Atg5^{f/f}$) mice. Atg5 is an essential autophagy gene, which conjugates with Atg12 and interacts with Atg16 to form a complex, serving as an E3 ubiquitin ligase that regulates the conjugation of LC3 with phosphatidylethanolamine (PE). This process is essential in autophagosome membrane elongation. Therefore, *Atg5* deletion directly leads to autophagy deficiency. Preadipocytes with *Atg5* knockdown and primary mouse embryonic fibroblasts (MEFs) from *Atg5* KO mice show impaired adipogenesis (Singh et al. 2009). *Atg5* KO embryos and neonatal pups have fewer subcutaneous fat cells (Baerga et al. 2009). These results suggest that Atg5 and possibly Atg5-mediated autophagy is required for normal adipogenesis process.

Using A-Atg5 KO mice, I aimed to determine the effect of targeted autophagy deficiency on adipose tissue, and its consequent impact on response to alcohol-induced adipose atrophy and liver injury/steatosis.

2.3 Materials and Methods

Animals. *Adipoq-Cre* mice and Atg5$^{f/f}$ or Raptor $^{f/f}$ mice (backcrossed to C57BL/6J background) were crossbred to produce A-Atg5 KO mice and A-Raptor KO mice. The Cre- littermates were used as WT. Male and female (8- to 12-week-old) mice were used for ex vivo lipolysis assays, and only male mice (8- to 12-week-old) were used for chronic-and-binge ethanol model (Bertola et al. 2013). In brief, mice were acclimated to the Lieber-DeCarli liquid diet (F1259SP, Bio-serv) for 5 days, and then given ethanol (Decon)-containing (5%, v/v) liquid diet (F1258SP, Bio-serv) supplemented with maltose dextrin (Bio-serv) for 10 days. In the morning of the 15th day (i.e. day 10 since ethanol feeding started), the mice were orally gavaged with ethanol (5 g/kg) (ETOH group). Mice administered with calorie- and volume- matched control liqud diet and maltose dextrin gavage were used as control (CTRL group). Body weight was monitored every two days. Food intake was recorded and matched daily after alcohol feeding started. Eight hours after gavage, mice were euthanized, and blood, epididymal WAT (eWAT), retroperitoneal WAT (rWAT), subcutaneous WAT (sWAT), interscapular BAT (iBAT) and liver tissues were collected for following assays. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Protein Extraction and Western Blot. Fresh tissues were snap frozen in liquid nitrogen and then homogenized in ice-cold RIPA buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)], followed by centrifugation at 12,000 g x 15 min at 4□. The RIPA buffer was supplemented with protease inhibitor cocktail (BioTool). After centrifugation, the lipid layer was removed, supernatant was collected, and protein concentration was determined by BCA assay (Thermo). Samples were mixed with SDS loading buffer containing dithiothreitol (DTT)

and incubated at 95 °C for 10 min unless otherwise indicated. For non-heat-denaturing, non-reducing conditions, samples were mixed with SDS loading buffer without DTT and were not heated. Thirty microgram of protein was separated by SDS polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were incubated in Ponceau S solution (Sigma) for 5 min if needed, and neutralized with 0.1M NaOH and washed in Millipore water before blocking. Membranes were blocked in 5% milk in Tris-buffered saline with 0.1% Tween (TBST) at RT for 1 h, and then incubated with primary antibodies and secondary antibodies prepared in 5% milk in TBST. Signals were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermal) and/or Immobilon Western HRP Substrate (Millipore). The following antibodies were used (Table 6). Anti-LC3 antibody was generated as previously described (Ding et al. 2010). Densitometry was analyzed with Image J software (National Institute of Health, USA). Band densities for the proteins of interest were normalized to β-actin unless otherwise indicated.

Table 6 Summary of antibody information in Chapter II

Antibody	**Company**	**Catalog No.**
4E-BP1	Cell Signaling	9452
phospho-4E-BP1 (Ser65)	Cell Signaling	9451
AKT	Cell Signaling	9272
phospho-AKT (Ser473)(D9E)	Cell Signaling	4060
Atg5	Santa Cruz	sc-33210
β-actin	Sigma	A5441
CYP2E1	Abcam	Ab28146
FGF21	Abcam	ab64857

FoxO1	Cell Signaling	2880S
phospho-FoxO1 (Ser256)	Cell Signaling	9461S
GAPDH	Cell Signaling	2118
GSK-3β	Cell Signaling	5676
phospho-GSK-3β	Cell Signaling	5558
OXPHOS cocktail	Abcam	ab110413
p62/SQSTM1	Santa Cruz	sc-28359
UCP1	Abcam	ab10983

Protein Oxidation Detection. Protein oxidation/carbonylation was detected with Oxyblot kit (Millipore) following the manufacturer's instructions. In brief, 5 μL of sample containing 20 μg of protein was denatured by 5 μL of 12% SDS. Samples were incubated with 10 μL of 1x 2-4-dinitrophenyl hydrazine (DNPH) solution at RT for 15 min, and samples incubated with 1x Derivation-Control Solution was used as a negative control. Later, 7.5 μL of Neutralization Solution was added to stop the derivatization, and 1.5 μL 2-mercaptoethanol was added as reducing agent. Then the samples were loaded to SDS polyacrylamide gel for electrophoresis. The following steps were similar to common Western Blot procedures as mentioned above, except that the designated primary antibody (specific to the DNP moiety) and secondary antibody (goat anti-rabbit IgG) in the kit were used.

RNA Extraction and Real-time Polymerase Chain Reaction (PCR). For liver tissue, RNA was isolated with TRIzol Reagent (Invitrogen) following the manufacturer's protocol. For adipose tissue, RNA was isolated with PureLink RNA Mini kit (Invitrogen) with or without TRIzol following the manufacturer's protocol. RNA concentration and quality was determined by

spectrophotometer. One to three nanograms of RNA was used for cDNA synthesis with reverse transcription kit (Fermentas). mRNA levels of target genes were determined on an Applied Biosystems Prism 7900HT real-time PCR instrument (ABI, Foster City, CA) using Maxima SYBR green/rox qPCR reagents (Fermentas). *18s* and *36b4* were used for normalization in liver samples and adipose tissue samples, respectively. The primer sequences were described in Table 7.

Table 7 Summary of mouse primer sequences for qPCR

Gene	Forward Sequences (5' - 3')	Reverse Sequences (5' - 3')
18s	GAGCGAAAGCATTTGCCAAG	GGCATCGTTTATGGTCGGAA
36b4	TAAAGACTGGAGACAAGGTG	GTGTACTCAGTCTCCACAGA
Adipor1	CTTGACGATGCTGAGACCAA	GCTGTGGGGAGCAGTAGAAG
Adipor2	GCCCAGCTTAGAGACACCTG	GCCTTCCCACACCTTACAAA
Cidea	CTCGGCTGTCTCAATGTCAA	GGGATGGCTGCTCTTCTGTA
Ccl2	ACATTCGGCGGTTGCTCTAG	ACATCCTGTATCCACACGGCAG
Cox8b	GAACCATGAAGCCAACGACT	GCGAAGTTCACAGTGGTTCC
Dio2	CAGTGTGGTGCACGTCTCCAATC	TGAACCAAAGTTGACCACCAG
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Fgf21	CTGGGGGTCTACCAAGCATA	CACCCAGGATTTGAATGACC
Il-1b	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG
Il-6	ACAAGTCGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC
Klb	GATGAAGAATTTCCTAAACCAGGTT	AACCAAACACGCGGATTTC
Ly6g	TGCGTTGCTCTGGAGATAGA	CAGAGTAGTGGGGCAGATGG
Prdm16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
Tnfa	CGTCAGCCGATTTGCTATCT	CGGACTCCGCAAAGTCTAAG
Ucp1	AGGCTTCCAGTACCATTAGGT	CTGAGTGAGGCAAAGCTGATTT

Histological Analysis. Fresh liver and fat tissues were fixed in 10% neutral-buffered formalin overnight, transferred to 70% ethanol for at least 24 h, embedded in paraffin wax, and cut into 5 μm sections. For general morphology, slides were stained with hematoxyline and eosin (H&E).

For eWAT adipocyte area quantification, at least 3 fields of 10x images of each mice were analyzed in each group using Adipocyte Tools (Montpellier Ressources Imagerie) with Image J. For BAT LD quantification, at least 5 fields of 20x images of each mouse were analyzed using Image J.

Immunohistochemistry Staining. Paraffin-embedded slides were rehydrated, quenched of endogenous peroxidase and blocked. Then the tissues were incubated with primary antibodies (1:1000) overnight at 4°C, and then incubated with biotinylated secondary antibodies. For neutrophil staining, slides were incubated anti-Ly6B2 antibodies (1:200) overnight at 4°C, and then incubated with biotinylated secondary antibody. For quantification, 10 fields of 20x images were randomly picked for each sample.

Electron Microscopy. Fresh tissues and cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) followed by 1% OsO4. After dehydration, thin sections were stained with uranyl acetate and lead citrate. Digital images were captured using a JEM 1016CX electron microscope (JEOL, Peabody, MA).

Liver Oil Red Staining. One gram of Oil Red O (Sigma) was dissolved in 200 ml isopropanol to make Oil Red O stock solution. The stock solution and Millipore water (v/v=6:4) was mixed well and filtered through 125mm filter paper (Whatman) to make working solution before use. Liver tissues were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4°C, transferred to 20% sucrose overnight at 4°C, and embedded in O.C.T. Compound (Fisher). For microscopy, tissues

were cut into 5 μm slides using a cryostat. The cryostat sections were dried at 37°C and washed twice with PBS, incubated in 60% 2-propanol for 1 min, dried again at 37 °C, and incubated with Oil Red O working solution for 10-15 min at RT. Then the slides were washed with 60% 2-propanol for 2-5 min, one time and with PBS, three times. After counterstaing with hematoxylin for 5 sec, the slides were washed with RO water for at least 3 min and mounted with glycerol in PBS (v/v=5:1). Images were captured within the same day.

Liver TG and Cholesterol Analysis. Liver TG and cholesterol levels were determined as previously reported (Buettner et al. 2000). Frozen liver tissue (20 to 50 mg) was ground in liquid nitrogen using a mortar and a pestle. Then the homogenate was incubated with 1 mL of chloroform/methanol (v/v=2:1) with vigorous shaking for 1 h at room temperature (RT). Then 200 μL of Millipore water was added into the mix and centrifuged at 3000 g for 5 min. The lower lipid phase was collected and dried in fume hood overnight, and the pellet was dissolved in 60 μL of tert-butanol and 40 μL of Triton X-114/methanol (v/v=2:1) mix. Liver TG and cholesterol levels were measured with colorimetric assay kits following the manufacturer's instructions (Pointe Scientific) and normalized to tissue weight. The tert-butanol and Triton X-114/methanol mixture was used as a blank control.

Serum Biochemical Analysis. Blood samples were collected via cardiac puncture, sat on ice for at least 30 min, centrifuged at 3,000 min at 4°C for 10 min, and the supernatant was collected as serum samples. Serum alanine aminotransferase (ALT) and aspartase aminotransferase (AST) levels were measured with fluorometric assay kits (Pointe Scientific). Serum TG and cholesterol levels were measured with colorimetric assay kits (Pointe Scientific). Serum FFA level was

measured with a fluorometric assay kit (BioVision). Serum free glycerol level was measured with a colorimetric assay kit (Sigma-Aldrich). Serum β-hydroxybutyrate (β-HB) level was measured with a colorimetric assay kit (BioVision). Millipore water was used as a blank control. All assays were performed following the manufacturer's instructions on a spectrometer unless otherwise indicated.

Enzyme-linked Immunosorbent Assay (ELISA). Serum samples were collected as described above. 2.5 μL of serum was used to measure the adiponectin level with a commercial kit (Millipore) following the manufacturer's protocol. 10 μL of serum was used to measure the FGF-21 level with a commercial kit (R&D) following the manufacturer's protocol.

***Ex Vivo* Lipolysis Assay.** *Ex vivo* lipolysis assay was performed as previously described (Zhang et al. 2015). In brief, after mice were euthanized, eWAT and sWAT tissues were collected, cut into smaller pieces, and weighed (around 50mg per piece). Each piece was placed in 12-well plate and incubated in Dulbecco's Modified Eagle Medium (DMEM) with or without isoproterenol on an orbital shaker for 1 h. The free glycerol released in medium was measured and normalized to tissue weight.

Statistical Analysis. Experimental data were subjected to Student's *t* test or One-way ANOVA where appropriate. Error bars presented standard error. A $p < 0.05$ was considered significant.

2.4 Results

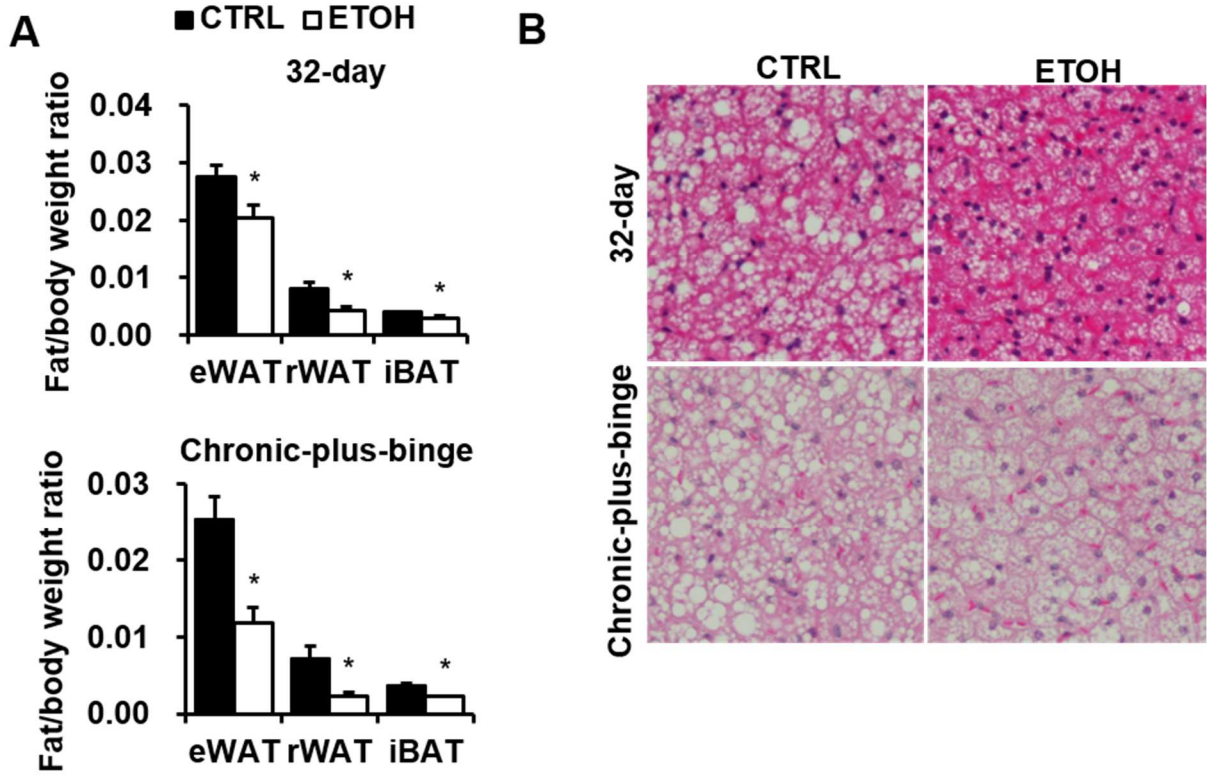
2.4.1 Chronic-plus-binge Alcohol Treatment Leads to Adipose Atrophy Associated with Inhibited Akt/mTOR Signaling and Increased Autophagy

Eight-week alcohol feeding in mice decreased fat tissue size and weight (Sun et al. 2012, Zhong et al. 2012). The effect of chronic-plus-binge alcohol exposure on adipose tissue was hardly studied. We confirmed that the 32-day alcohol feeding significantly decreased the weight of eWAT, rWAT and iBAT (Fig.2.4.1A), and decreased the LD size of iBAT (Fig.2.4.1B). Moreover, we found that the chronic-plus-binge treatment significantly decreased adipose tissue weight and the LD size of iBAT (Fig.2.4.1A, B) in C57BL/6J WT mice, suggesting that chronic alcohol and chronic-plus-binge treatment leads to adipose atrophy.

Adipose-specific *mTOR/Raptor* KO mice develop lipodystrophy (Lee et al. 2016, Shan et al. 2016). In our hands, we confirmed that mice with adipose-specific *Raptor* deletion had significantly smaller white and brown adipose tissues, a phenotype similar to alcohol-treated mice (Fig.2.4.2). Thus we sought to determine if alcohol exposure leads to adipose atrophy involving mTOR inhibition. We found that in eWAT after chronic-plus-binge treatment, both the levels of upstream phosphorylated protein kinase B (Akt/PKB) and total Akt proteins decreased (Fig.2.4.1C). Moreover, the levels of total and phosphorylated levels of glycogen synthase kinase-3 beta (GSK3β) and FoxO1, two downstream signaling nodes of Akt, also decreased after chronic-plus-binge alcohol. Both the total and phosphorylated levels of 4EBP1, the target substrate of mTOR, also decreased after chronic-plus-binge alcohol. These data suggest that chronic-plus-binge alcohol treatment inhibits Akt/mTOR signaling in mouse adipose tissue.

mTOR inhibition is a common trigger of autophagy activation. Previous studies reported that adipose-specific *Atg5* or *Atg7* KO mice had smaller adipose tissue (Baerga et al. 2009, Zhang et al. 2009). Therefore, we thought to answer if alcohol exposure leads to adipose atrophy involving the autophagy pathway. Interestingly we found that in eWAT after chronic-plus-binge treatment, the levels of both LC3-II and p62 and decreased, (Fig.2.4.1D, E). p62 is a well-known autophagy substrate protein that is often degraded by increased autophagy. LC3-II decorateds the outer and inner membrane of autophagosomes, but LC3-II is also degraded by autolysosomes. Therefore, it is likely that decreased p62 and LC3-II in adipose tissue after chronic-plus-binge alcohol is due to increased autophagic flux.

Figure 2.4.1



(See figure legend on next page.)

Figure 2.4.1 (Cont.)

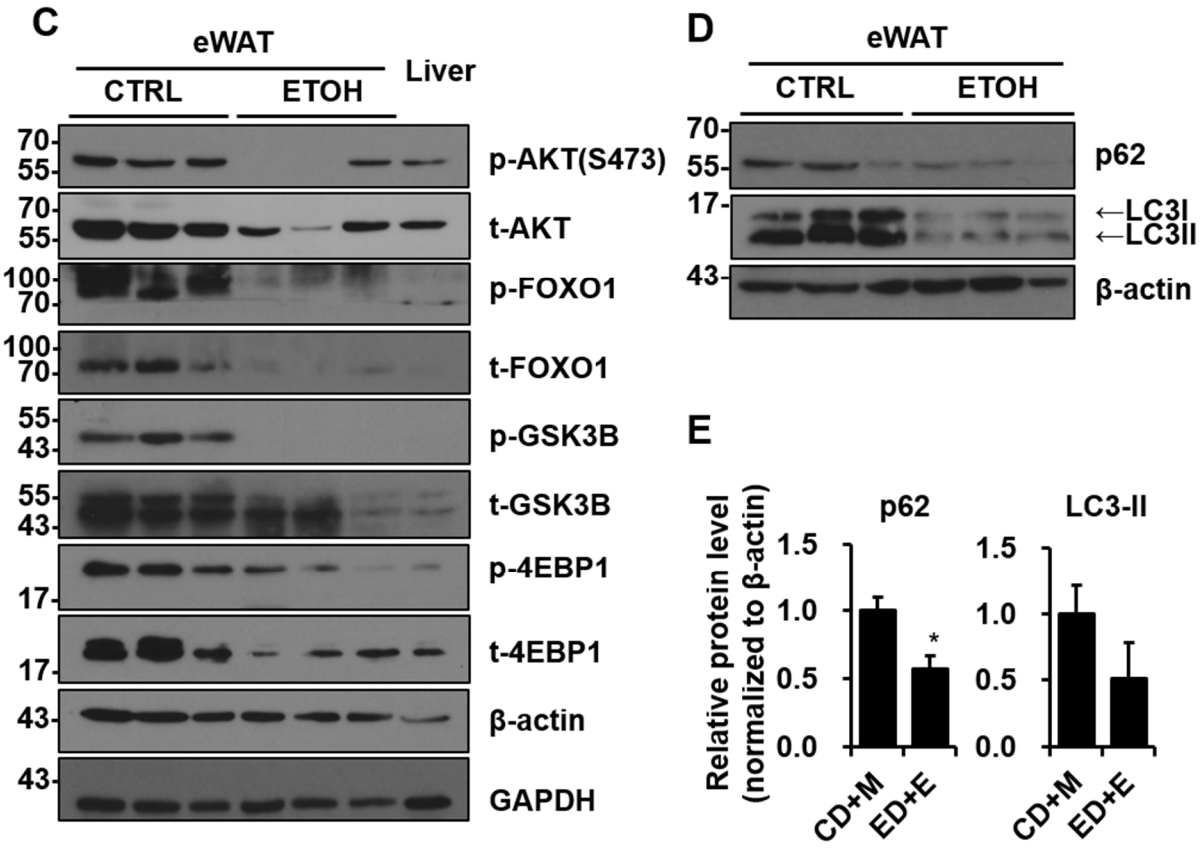


Figure 2.4.1 Alcohol induced adipose atrophy is associated with inhibited mTOR and increased autophagy in epididymal white adipose tissue.

C57BL/6J WT mice were treated with either 32-day chronic alcohol feeding, and fat tissues were collected on the last day; or 10-day chronic-plus-binge alcohol treatment, and fat tissues were collected 8 h after binge. (A) Adipose tissue weight quantification. (B) representative H&E staining of iBAT tissues of C57BL/6J WT mice after alcohol treatment (32-day: *n*=4 mice per group; chronic-plus-binge: *n*=4-8 mice per group). Magnification=200x. (C)(D) Representative western blot of eWAT and liver of C57BL/6J WT mice with chronic-plus-binge treatment. β-actin and GAPDH was used as loading control. (E) Densitometry of (D) (*n*=5 mice per group). CTRL: control treatment group; ETOH: ethanol treatment group. Data shown are mean±SEM. **p*<0.05(CTRL vs ETOH) by Student's *t* test.

Figure 2.4.2

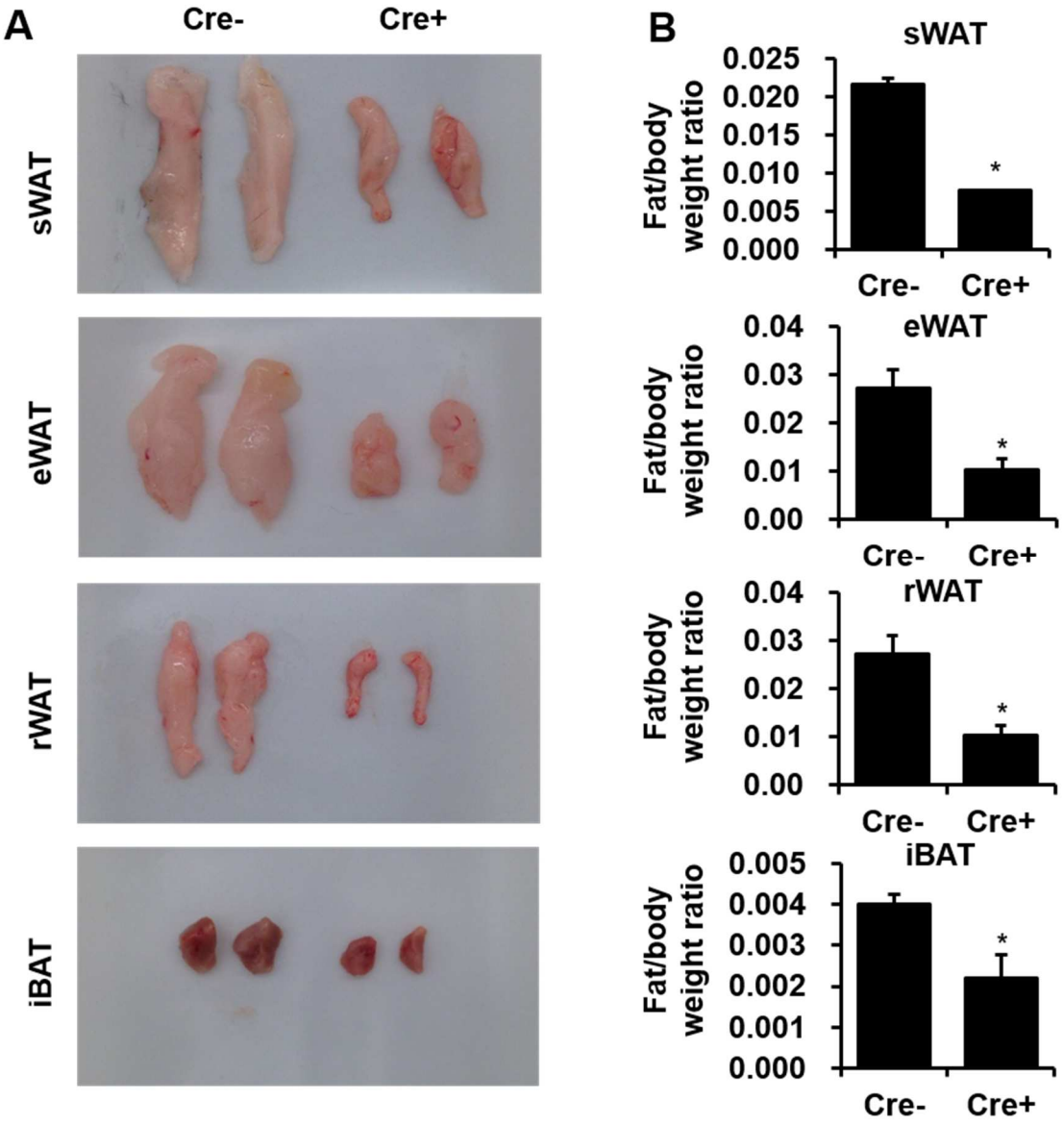


Figure 2.4.2 Adipose-Raptor deficient mice developed adipocyte atrophy.
Fat samples were collected from Raptor$^{f/f}$, *Adipoq*-Cre- and -Cre+ mice (A) Representative epididymal (eWAT), subcutaneous (sWAT), retroperitoneal (rWAT) white adipose tissue and interscapular brown adipose tissue (iBAT). (B) Quantification of adipose tissue weight *(n*=3-7 mice per group). Data shown are mean±SEM. **p*<0.05 (Cre- vs Cre+) by Student's *t* test.

2.4.2 A-Atg5 KO Mice Are Less Sensitive to Chronic-plus-binge Alcohol-induced Adipose Atrophy

To study adipose autophagy, we crossed *Adipoq-Cre* mice with $Atg5^{f/f}$ mice in which LoxP sites flank exon 3 of Atg5 (Fig.2.4.3A). We first examined if *Atg5* deletion was specific and potent in adipose tissues. Atg5-Atg12-Atg16 complex functions as an E3 ligase essential for the lipidation of LC3. When Atg5 is absent, LC3-II formation is interrupted, so the autophagy process is halted and the adaptor protein p62 accumulates. In A-Atg5 KO mouse, the level of Atg5-Atg12 conjugate decreased in eWAT, sWAT and iBAT along with increased p62 and LC3-I (Fig.2.4.3B), suggesting deficient autophagy in A-Atg5 KO mice. Noticeably, the basal level of autophagy proteins and even internal controls were very different among different adipose tissues. We also examined liver and skeletal muscle since they are crucial in nutrient metabolism and tightly associated with adipose tissues. Atg5-Atg12, p62 and LC3 were not affected in liver or skeletal muscle of A-Atg5 KO mice, suggesting that the deletion of *Atg5* is specific in adipose tissues.

Figure 2.4.3

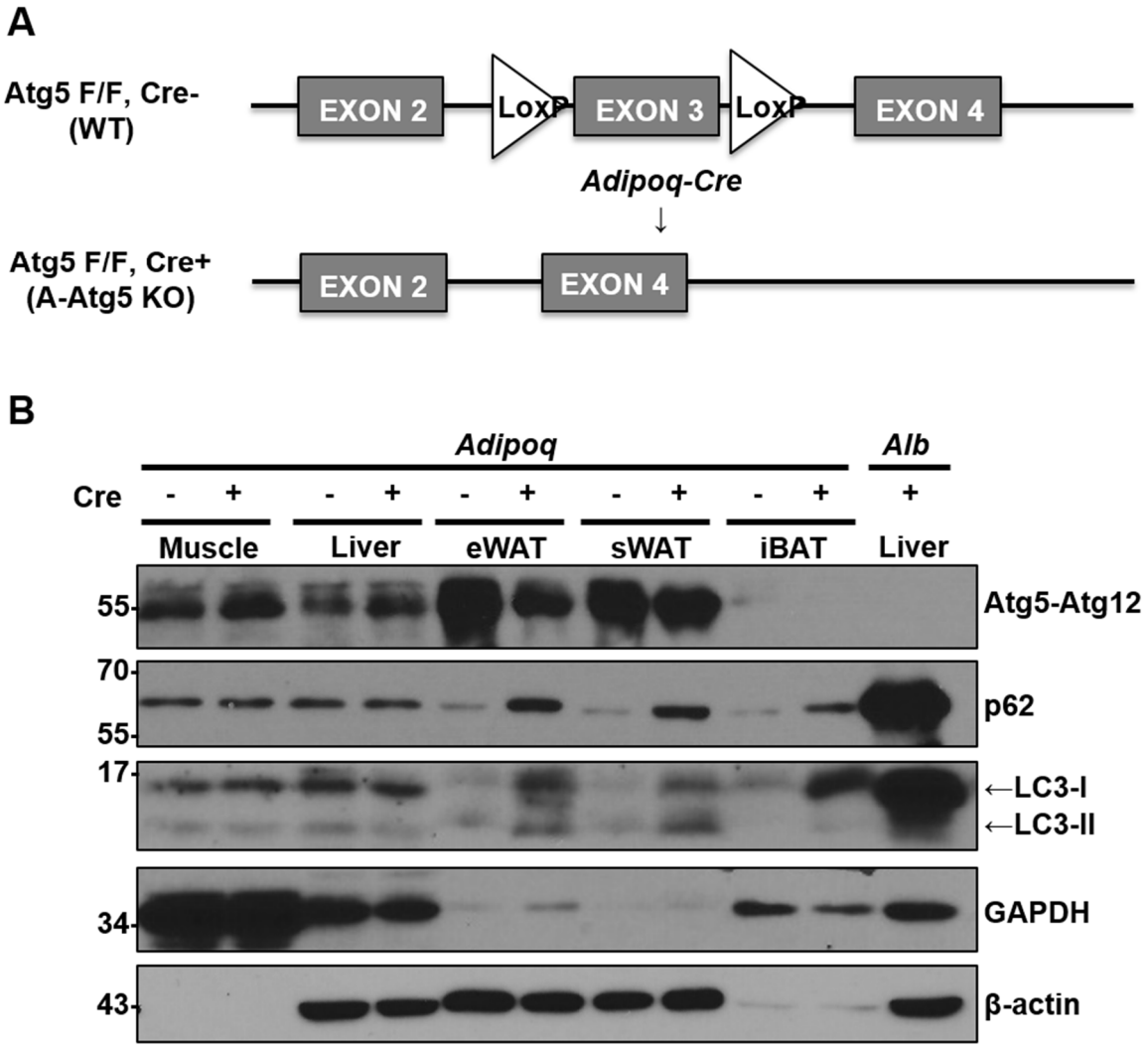
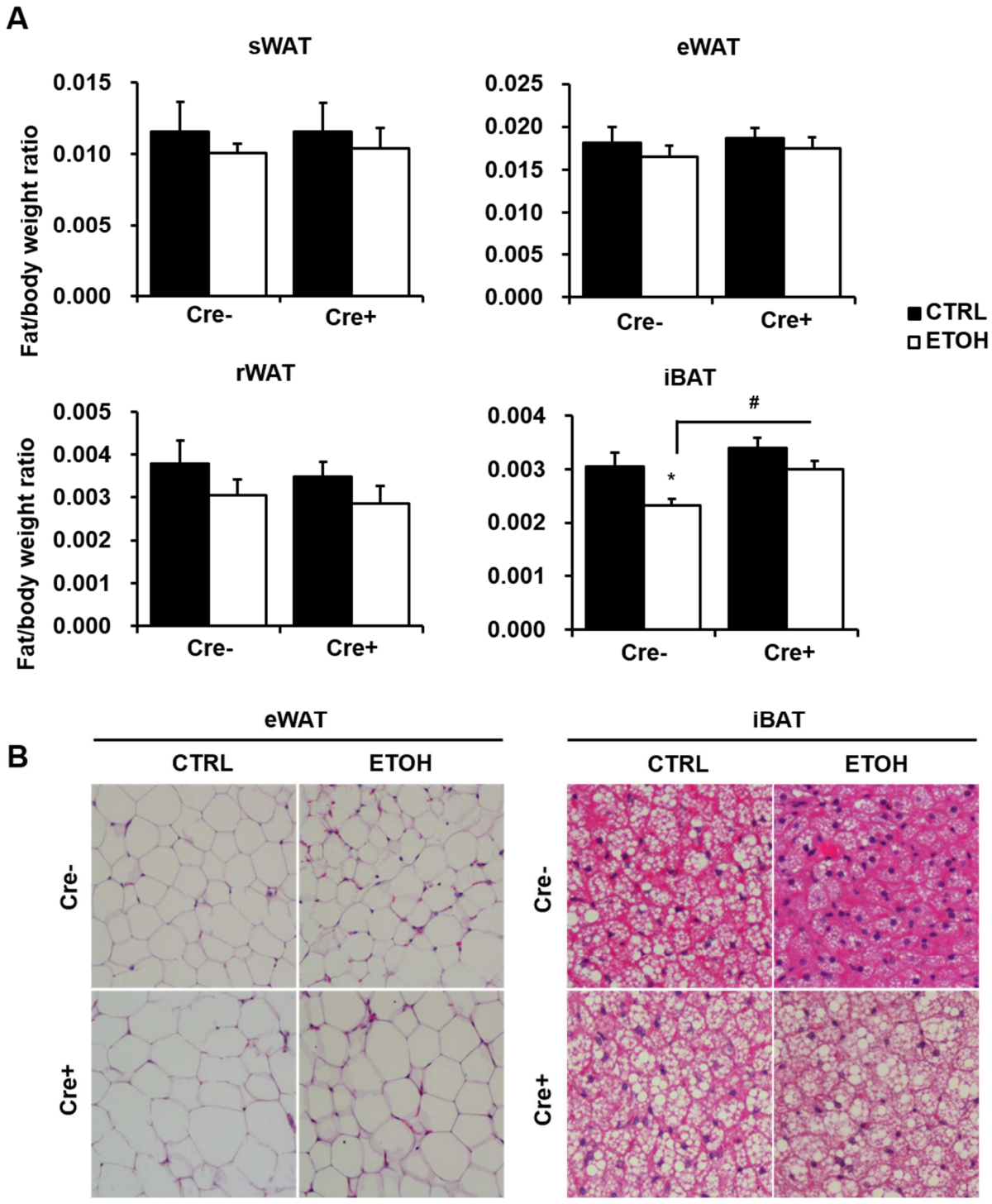


Figure 2.4.3 Generation and validation of adipose tissue-specific *Atg5* knockout mice.

(A) Targeting strategy for conditional *Atg5* deletion. (B) Skeletal muscle, liver, eWAT, sWAT and iBAT tissues were collected from $Atg5^{f/f}$, *Adipoq*-Cre- and -Cre+ mice, and protein was extracted and subjected to western blot. Liver protein lysate from Atg5 F/F, *Albumin-Cre*+ mouse was used as negative control. GAPDH and β-actin were used as internal control.

To our surprise, the ratio of various adipose tissues vs body weight in A-Atg5 KO mice had no significant difference when compared with WT mice (Fig.2.4.4A). In response to chronic-plus-binge alcohol, WT mice had slightly reduced WAT mass (by 10-20%) and significantly reduced iBAT mass, while the iBAT mass of A-Atg5 KO mice was significantly higher than WT mice. The size of adipocyte/LD in eWAT and iBAT decreased after alcohol treatment in WT mice, but it remained unchanged in A-Atg5 KO mice (Fig.2.4.4B). In WT mice, 25% of eWAT adipocytes had an area of 400-1600 pixel/100x field, while in KO mice only about 40% of eWAT adipocytes were in the 400-1600 range and fewer adipocytes were in the larger area range (Fig.2.4.4C). In addition, alcohol decreased LD size per field by ~40% and per cell by ~50% in iBAT in WT but not in A-Atg5 KO mice (Fig.2.4.4D). In our hands, mouse body weight usually drops a little in the first few days since alcohol feeding starts (counted as Day 1) and gradually restores once the mice are acclimated to ethanol-containing liquid diet. The body weights on Day 1 and the last day of feeding (Day 10) were not significantly different between WT and KO mice (Fig.2.4.5A). The average food intake from Day1 to Day 10 was also not changed in KO mice (Fig.2.4.5B). Taken together, A-Atg5 KO mice presented resistance to alcohol-induced adipose atrophy especially in WAT, and this was not due to increased bodyweight or nutrient intake.

Figure 2.4.4



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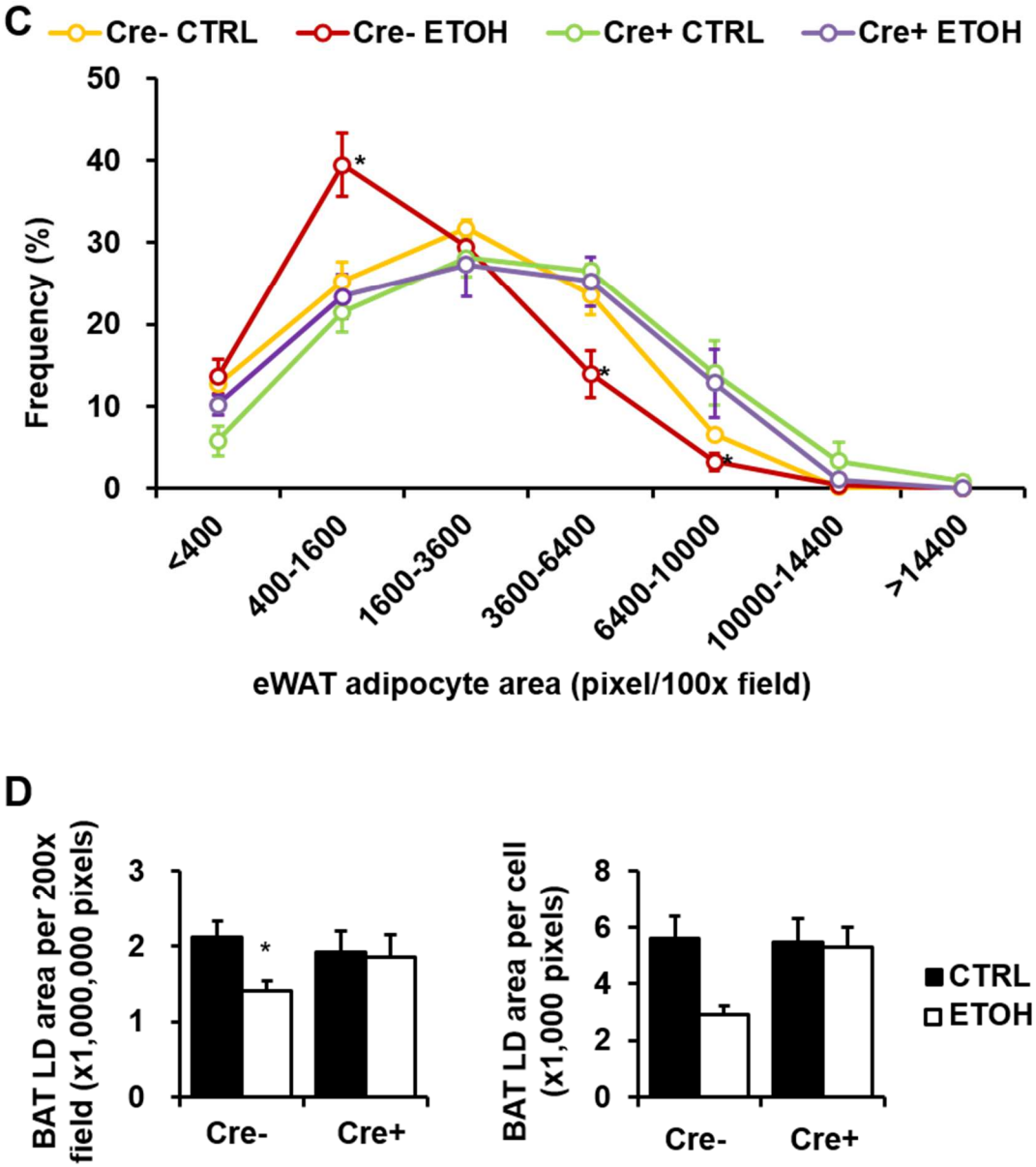


Figure 2.4.4 Adipose-Atg5 deficient mice were partially resistant to alcohol-induced adipocyte atrophy.

WT and A-Atg5 KO and mice were treated with chronic-plus-binge alcohol, and fat samples were collected 8h after binge. (A) Quantification of epididymal (eWAT), subcutaneous (sWAT), retroperitoneal (rWAT) white adipose tissue and interscapular brown adipose tissue (iBAT) weights (n=5-21 mice per group). (B) Representative H&E staining of eWAT and iBAT tissues (magnification=20x). (C) Distribution of eWAT adipocyte area in H&E staining (n=3-6 mice per group). (D)Quantification of iBAT LD area in H&E staining (n=4-8 mice per group). Data shown are mean±SEM, *p<0.05 (CTRL vs ETOH), #p<0.05 (Cre- vs Cre+) by One-way ANOVA.

Figure 2.4.5

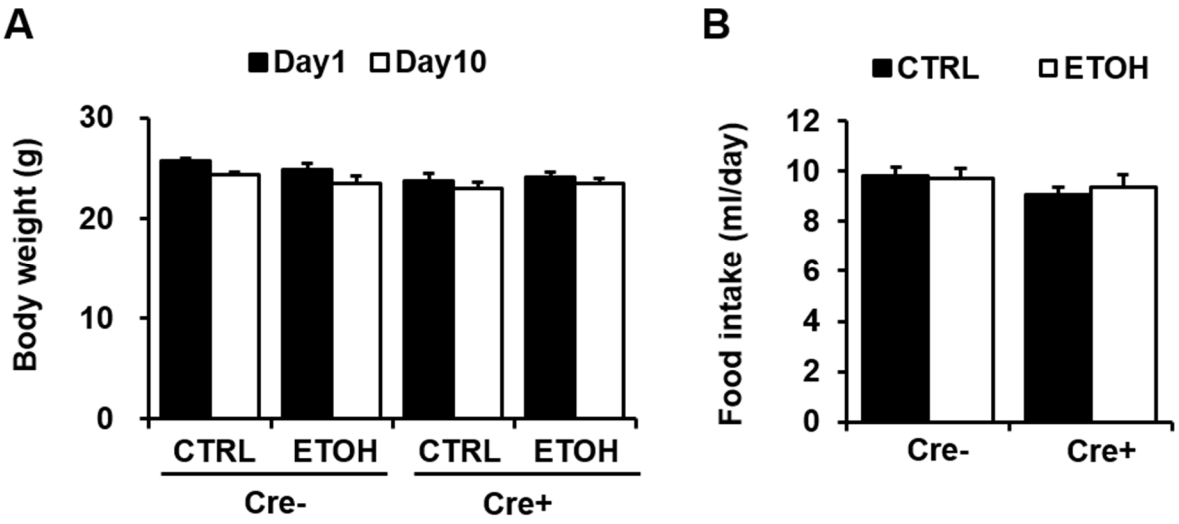


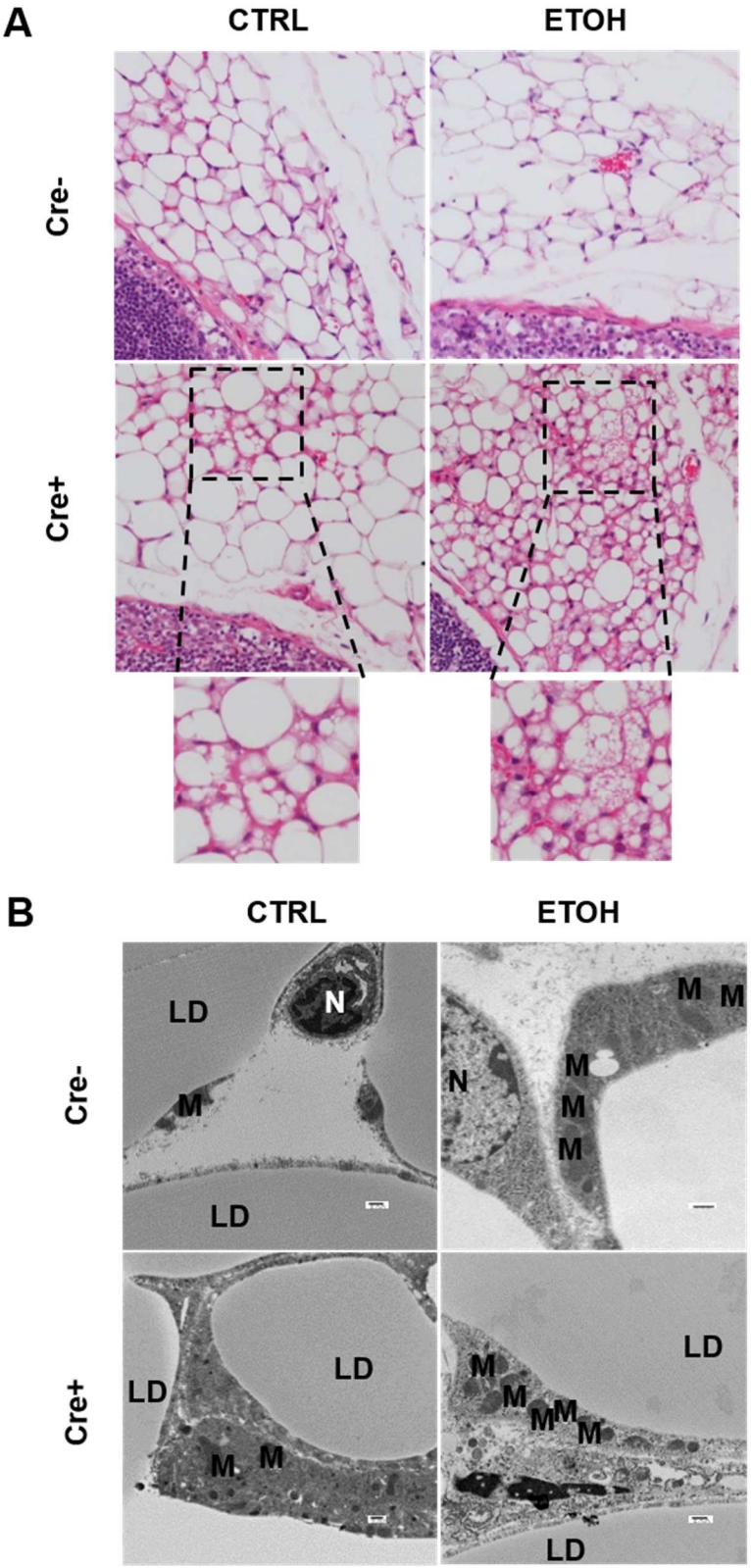
Figure 2.4.5 Adipose-Atg5 deficient mice had similar body weight and food intake to WT mice.

WT and A-Atg5 KO mice were treated with chronic-plus-binge alcohol, and body weight and food intake were recorded. (A) The average body weight on Day 1 (since alcohol feeding started) and Day 10 (*n*=8-12 mice per group). (B) The average food intake (*n*=8-12 mice per group). Data shown are mean±SEM.

2.4.3 A-Atg5 KO Mice Have Increased sWAT Browning

Autophagy-mediated degradation of mitochondria is a hallmark feature of the transition of browning adipocytes to white adipocytes (Altshuler-Keylin et al. 2016). Therefore, lack of autophagic removal of mitochondria may favor the browning of white adipocytes. We found depots of adipocytes with multilocular LDs around lymph nodes in sWAT of A-Atg5 KO mice, which was further increased after chronic-plus-binge alcohol treatment. Multilocular LDs were rarely seen in WT mice (Fig.2.4.6A). In EM studies, there were increased mitochondria numbers in sWAT adipocytes of A-Atg5 KO mice after alcohol treatment (Fig.2.4.6B). Alcohol treatment increased the levels of UCP1 protein. The basal levels of UCP1 protein and mitochondria oxidative phosphorylation proteins markedly increased in sWAT of A-Atg5 KO mice compared with the matched wild-type mice (Fig.2.4.6C). However, the gene expression of genes that thought to be specially expressed in brown/beige adipocytes including *Ucp1*, *Prdm16*, *Cox8b*, *Dio2*, and *Cidea*, were not different between wild-type and A-Atg5 KO mice (Fig.2.4.6D).

Figure 2.4.6



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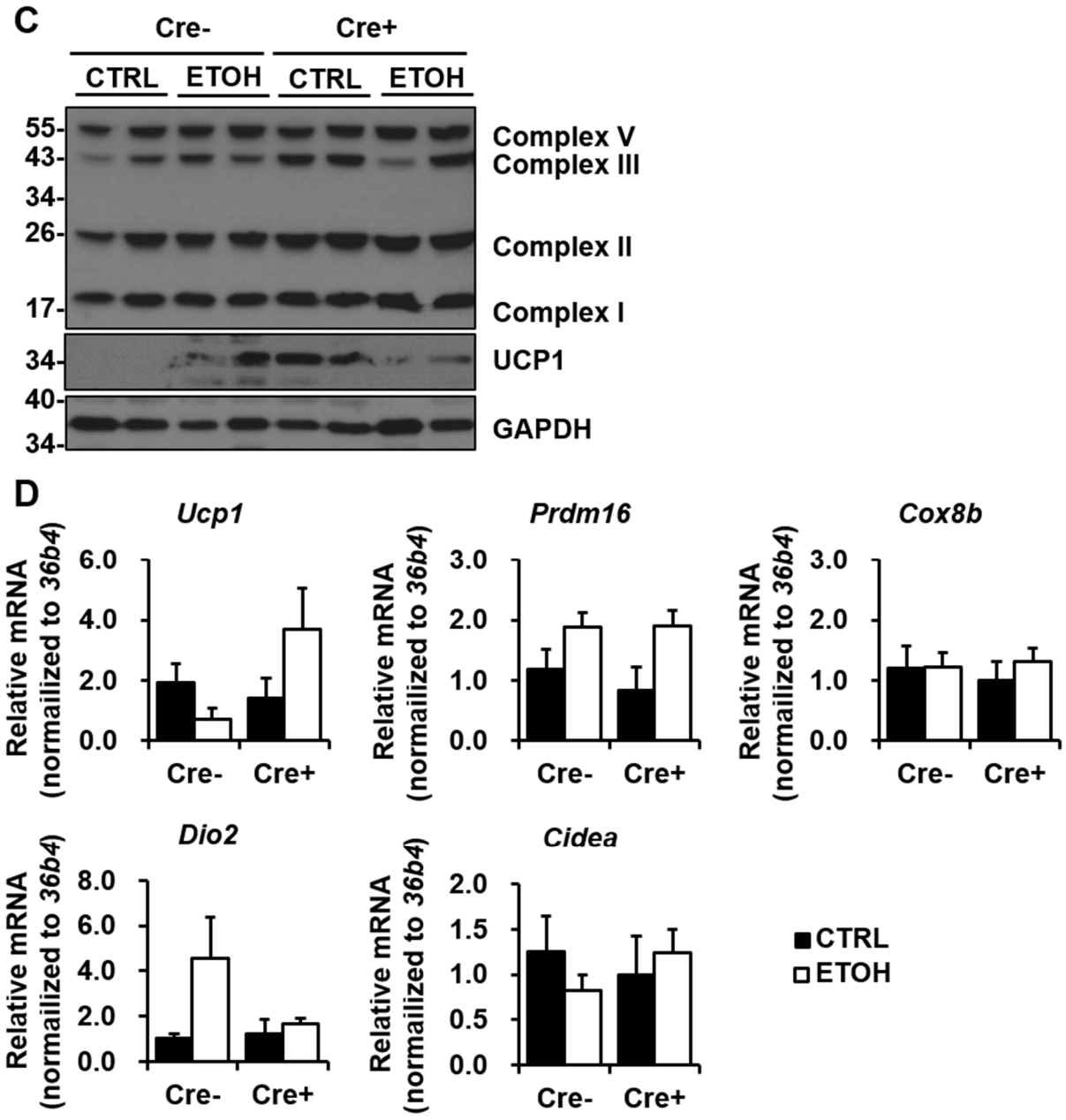

Figure 2.4.6 Adipose-specific Atg5 deficient mice had increased sWAT browning.
WT and A-Atg5 KO mice were treated with chronic-plus-binge alcohol, and sWAT samples were collected 8h after binge. (A) Representative H&E staining of sWAT. (B) Representative EM image of sWAT. (C) Representative western blot image. sWAT was subjected to protein extraction and western blot. GAPDH was used as internal control. (D) sWAT was subjected to mRNA extraction and qPCR (*n*=4-5 mice per group).

2.4.4 A-Atg5 KO Mice Are Protected from Alcohol-induced Liver Injury but Not Steatosis

We next examined alcohol-induced liver injury and steatosis in A-Atg5 KO mice. In WT mice, chronic-plus-binge alcohol treatment resulted in liver macrosteatosis and microsteatosis, i.e. visualization of big and small LDs, in H&E staining (Fig.2.4.7A). This observation was supported by increased Oil Red O staining of liver tissue (Fig.2.4.7B), with significantly increased liver/body weight ratio (Fig.2.4.7C) and liver TG (Fig.2.4.7D) in alcohol-treated mice compared with control mice. Liver cholesterol was slightly increased after alcohol (Fig.2.4.7D). These data suggest that chronic-plus-binge alcohol induces hepatic steatosis in mice. A-Atg5 KO mice had similar morphology of H&E and Oil Red O staining in response to alcohol. In addition, the changes of liver weight, levels of hepatic TG as well as cholesterol were also similar in A-Atg5 KO mice compared with WT mice. These data suggest that A-Atg5 KO mice do not affect alcohol-induced steatosis.

ALT and AST are enzymes usually found in liver but released into blood when hepatocytes are damaged. AST may also come from other tissues like heart and muscle. Though the level of serum ALT and AST can be affected by various mechanisms, they are two common markers indicating liver injury. In WT mice, chronic-plus-binge treatment led to elevated serum levels of ALT and AST significantly compared with control mice (Fig.2.4.7E) but no obvious cell death in liver H&E staining were observed (Fig.2.4.7F). In A-Atg5 KO mice, the serum levels of ALT and AST were significantly lower than WT mice after chronic-plus-binge alcohol. This suggests that chronic-plus-binge alcohol-induced liver injury is ameliorated in A-Atg5 KO mice.

Figure 2.4.7

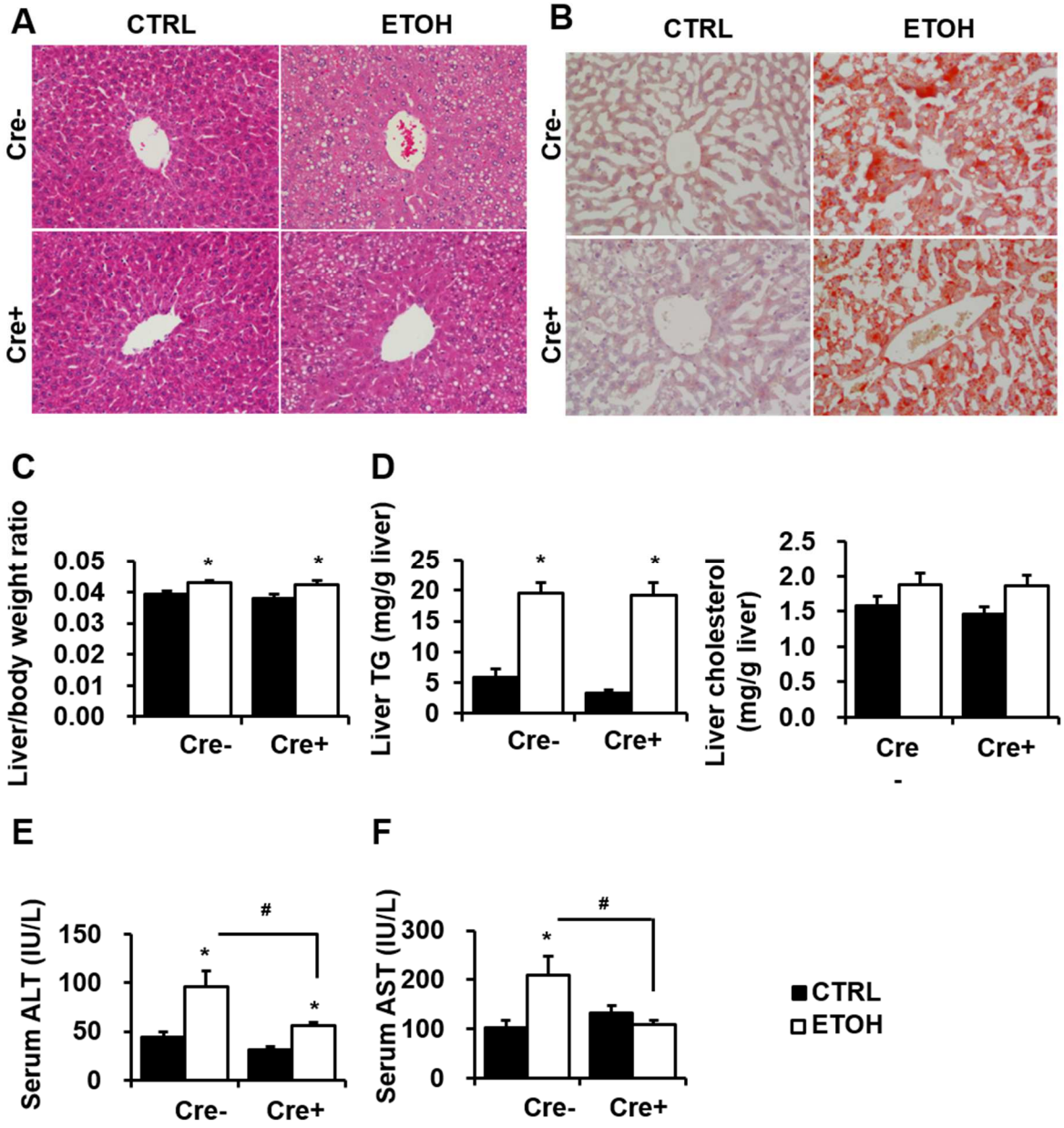

Figure 2.4.7 Adipose-Atg5 deficient mice were protected from alcohol-induced liver injury but not steatosis.

WT and A-Atg5 KO mice were treated with chronic-plus-binge alcohol, and serum and liver samples were collected 8h after binge. (A) Representative images of liver H&E staining (magnification=20x). (B) Representative images of liver Oil Red O staining (magnification=20x). (C) Quantification of liver weight (n=8-15 mice per group). (D) Liver tissue was subjected to lipid extraction, and TG and cholesterol levels were measured (n=8-12 mice per group). (E) Serum ALT levels were measured (n=8-20 mice per group). (F) Serum AST levels were measured (n=6-16 mice per group). Data shown are mean±SEM. *p<0.05 (CTRL vs ETOH), #p<0.05 (Cre- vs Cre+) by One-way ANOVA.

2.4.5 Protection against Alcohol-induced Liver Injury in A-Atg5 KO Mice Is Independent of Liver CYP2E1 Expression, ROS Generation, and Neutrophil Infiltration

In liver, alcohol metabolism is mainly catalyzed by ADH in the cytosol, CYP2E1 in microsomes and catalase in peroxisomes (Cederbaum 2012). The metabolism via CYP2E1 is largely responsible for ethanol-induced oxidative stress, and may contribute to ethanol-induced cell injury (Bardag-Gorce et al. 2000, Bradford et al. 2005, Lu and Cederbaum 2008). We asked if the liver protection in A-Atg5 KO mice was due to altered CYP2E1 metabolism and ROS production. We found that, alcohol treatment induced CYP2E1 proteins almost to the same levels in A-Atg5 KO and WT mice (Fig.2.4.8A, B).

The production of ROS promotes protein carbonylation, i.e. carbon monoxide introduced into protein side chains. The abundance of protein carbonyl groups like aldehyde and ketone is considered as a biomarker of oxidative damage (Fedorova et al. 2014). Carbonyl groups react with DNPH and generate DNP-derived proteins which can be detected via western blot using specific antibodies for DNP. We found alcohol treatment significantly increased carbonylation almost to the same levels in that in both WT and A-Atg5 KO mice (Fig.2.4.8C, D). Taken together, ethanol-induced CYP2E1 and ROS generation was not different in A-Atg5 KO mice compared with WT mice, and thus may not contribute to the protection against liver injury in A-Atg5 KO mice.

Figure 2.4.8

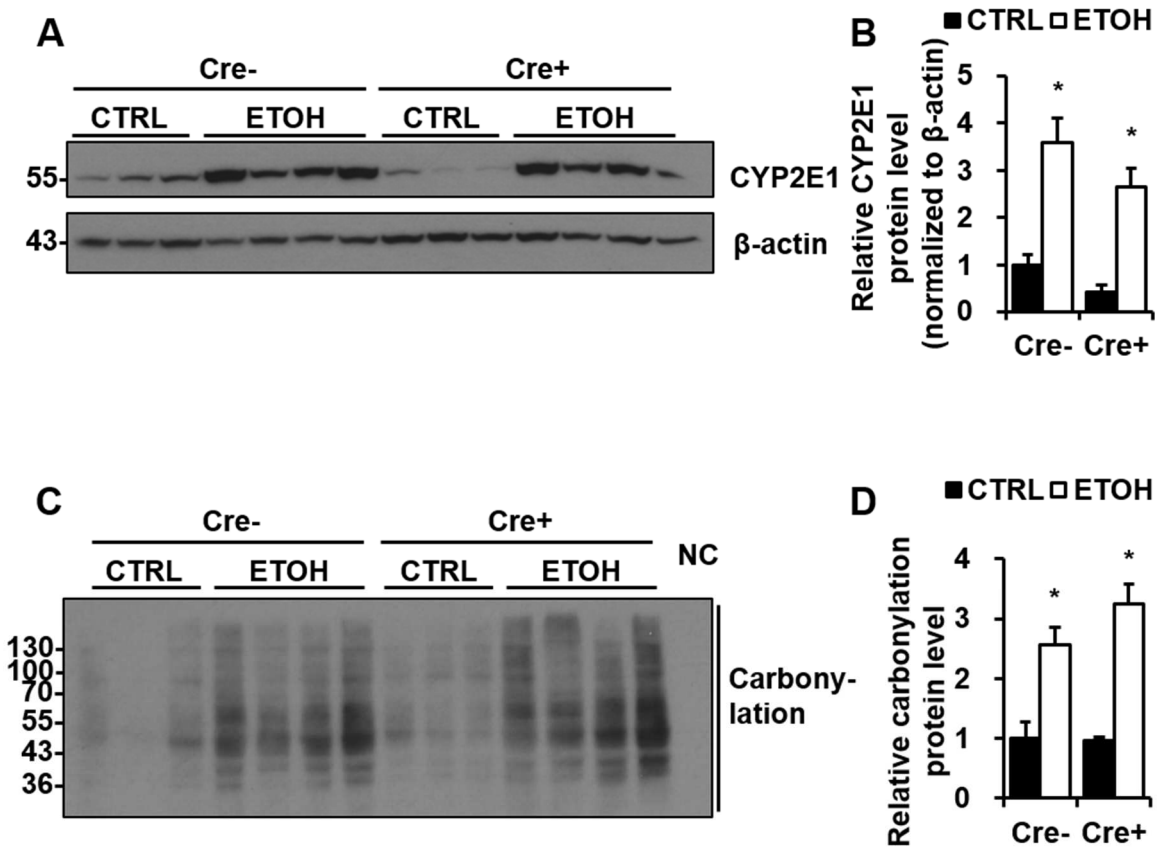

Figure 2.4.8 Adipose-Atg5 deficient mice had similar liver CYP2E1 and ROS generation after chronic-plus-binge alcohol.

(A) WT and A-Atg5 KO mice were treated with chronic-plus-binge alcohol, and liver samples were collected 8h after binge. Liver total protein was extracted and subjected to western blot. β-actin was used as internal control. (B) Densitometry of (A). (C) Liver total protein was subjected to western blot detecting carbonylation. NC: Negative control. (D) Densitometry of (C). Data shown are mean±SEM. *p<0.05 (CTRL vs ETOH) by One-way ANOVA.

Chronic alcohol-induced liver injury is associated with increased pro-inflammatory cytokines and chemokines and infiltration of inflammatory cells. We found in WT mice, chronic-plus-binge alcohol slightly increased neutrophil infiltration in the liver (Fig.2.4.9 A, B). Consistently, alcohol treatment significantly increased liver *Ly6g* mRNA level, a marker of neutrophils, monocytes and granulocytes (Fig.2.4.9 C). In A-Atg5 KO mice, we found that the neutrophil number seen in each field and the *Ly6g* mRNA level was similar to WT mice before and after alcohol. There was no significant induction of proinflammatory cytokines such as *Tnfa*, *Il-1b* or *Il-6,* or macrophage marker *F4/80* in liver after alcohol treatment. However, we found that alcohol treatment increased expression of *Ccl2*, a chemokine regulating recruitment of monocytes and macrophages, to almost 12 fold compared with control mice (Fig.2.4.9 C). In A-Atg5 KO mice, the mRNA levels of *Tnfa*, *Il-1b, Il-6* or *F4/80* were either similar to or higher than those in WT mice. However, the level of *Ccl2* mRNA was significantly lower in A-Atg5 KO mice than in WT mice after alcohol treatment. These data suggest that chronic-plus-binge alcohol may only lead to mild hepatic inflammation. Alcohol-induced induction of *Ccl2* is inhibited in A-Atg5 KO mice.

Figure 2.4.9

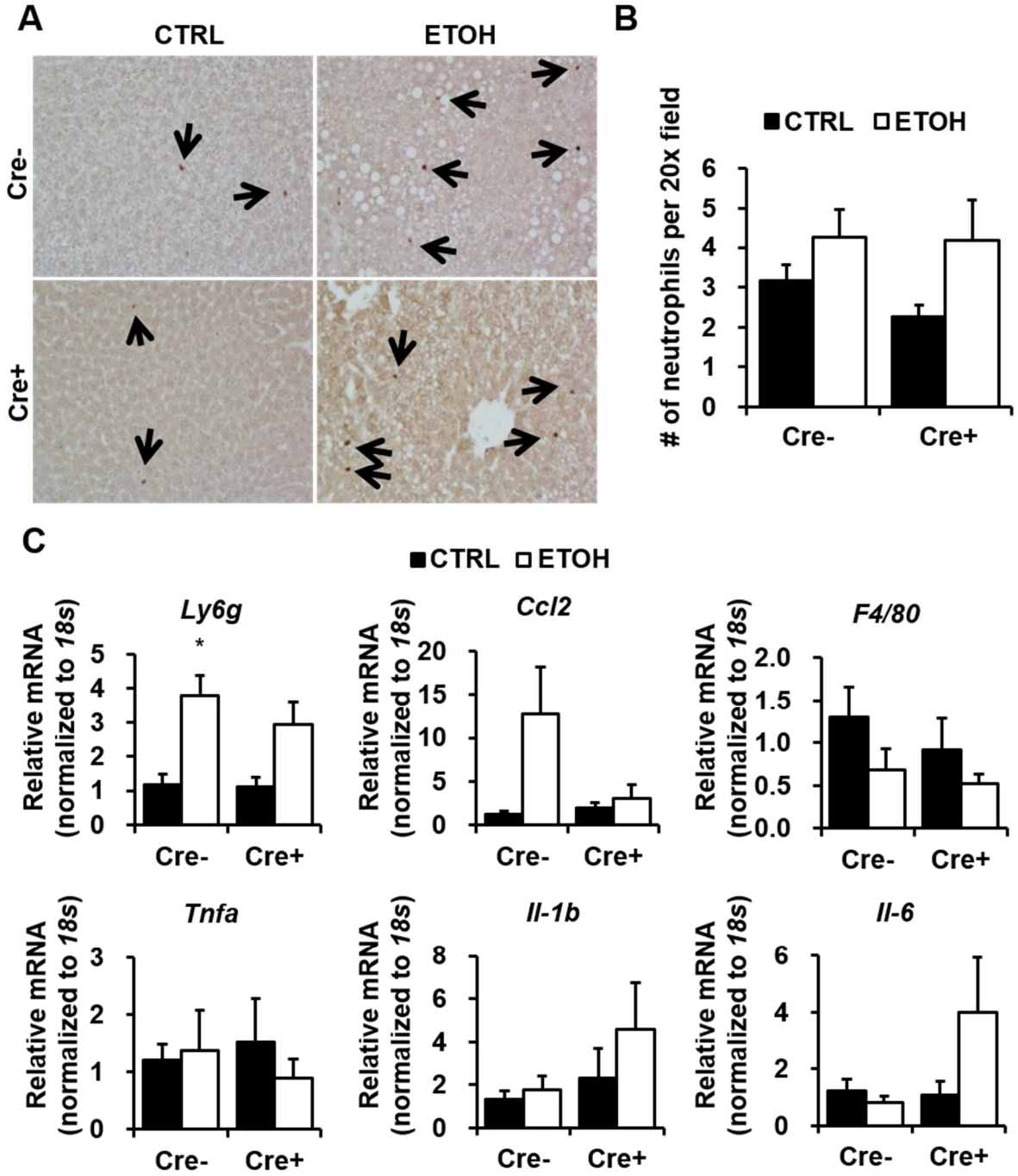

Figure 2.4.9 Adipose-Atg5 deficient mice were protected from chronic-plus-binge alcohol-induced liver injury independent of liver neutrophil infiltration.
WT and A-Atg5 KO mice were treated with chronic-plus-binge alcohol, and liver samples were collected 8h after binge. (A) Representative images of liver Ly6B2 immunochemistry staining (magnification=10x). Arrow indicates neutrophils. (B) Quantification of (A) (n=4-6 mice per group). (C) Liver was subjected to mRNA extraction and qPCR (n=4-5 mice per group). Data shown are mean±SEM. *p<0.05 (CTRL vs ETOH) by One-way ANOVA.

2.4.6 A-Atg5 KO Mice Have Similar Levels of Serum Lipids but Increased Basal Levels of Adiponectin and FGF21.

Lipolysis in adipose tissue is a stepwise breaking down of TG into FFAs and free glycerol mediated by lipases. Chronic alcohol exposure increases adipose lipolysis releasing FFAs and contributes to liver steatosis (Zhong et al. 2012). We determined whether chronic-plus-binge alcohol increased circulating lipids that may exacerbate liver steatosis (Fig.2.4.10A). In WT mice, alcohol treatment significantly increased serum FFA from 0.012 mM to 0.297 mM suggesting increased lipolysis (Fig.2.4.10B-D). Serum TG, cholesterol and free glycerol levels were not increased by alcohol. In A-Atg5 KO mice, the change in serum TG, cholesterol, FFA levels was similar to WT mice. The serum free glycerol level of KO mice was slightly lower than WT mice at the basal level. However, after alcohol treatment, the serum levels of glycerol increased to almost the same levels with WT mice. Taken together, these data suggest that A-Atg5 KO mice have similar serum lipids to WT mice after alcohol treatment.

FFAs released from adipocytes are taken up by hepatocytes and broken down into acetyl-CoAs in mitochondria for β-oxidation. Usually, acetyl-CoAs enter tricarboxylic cycle and generate ATPs. Under certain conditions like starvation, diabetic ketoacidosis and alcoholic ketoacidosis, acetyl-CoA metabolism shifts towards ketone body formation and generate metabolites including β-HB. Thus β-HB concentration partly reflects liver FA metabolism via β-oxidation. A recent study reported that β-HB protected chronic-plus-binge alcohol-induced liver injury via Hydroxycarboxylic acid receptor 2 (Hcar2) on liver macrophages (Newman and Verdin 2014, Chen et al. 2018). We found in WT mice, serum β-HB level was significantly increased to 3-fold after chronic-plus-binge alcohol treatment (Fig.2.4.10E). In A-Atg5 KO mice, serum β-HB had

higher basal level (50% increase), and was similar to WT mice after alcohol. This suggests that ketogenesis following FA β-oxidation in liver was similar in A-Atg5 KO and WT mice.

Figure 2.4.10

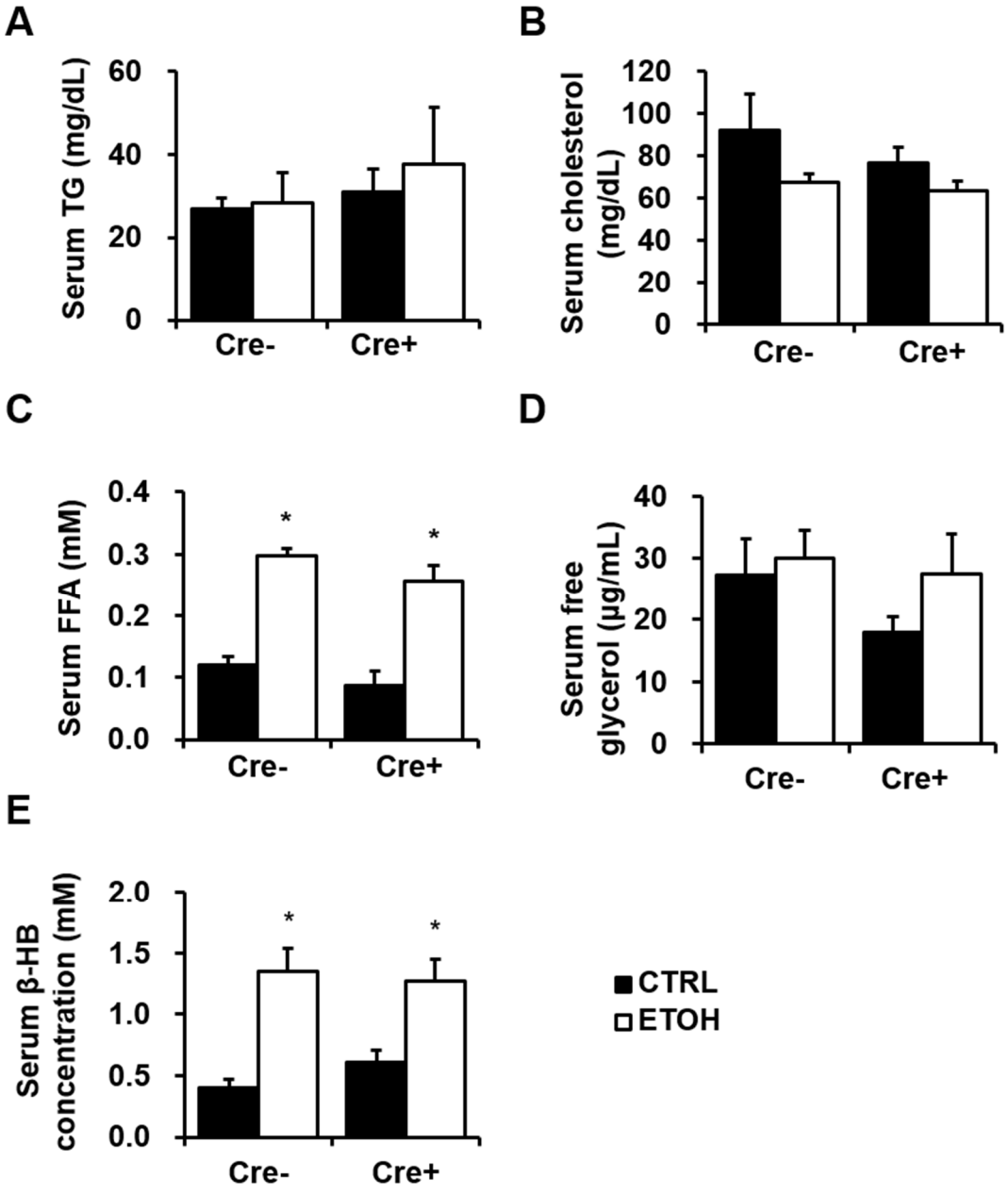

Figure 2.4.10 Adipose-Atg5 deficient mice had no change in serum lipids.
WT and A-Atg5 KO mice were treated with chronic-plus-binge alcohol, and serum samples were collected 8h after binge. (A) Serum TG and (B) cholesterol levels were measured (n=7-12 mice per group). (C) Serum FFA level was measured (n=7-11 mice per group). (D) Serum free glycerol level was measured (n=8-14 mice per group). (E) Serum β-hydroxybutyrate (β-HB) level was measured (n=7-12 mice per group). Data shown are mean±SEM, *p<0.05 (CTRL vs ETOH) by One-way ANOVA.

Adiponectin is secreted by adipose tissue and targets liver via receptors AdipoR1 and AdipoR2. Adiponectin regulates lipid and glucose metabolism and plays a protective role in various metabolic scenarios, and its response to alcohol exposure is still debatable. We found in WT mice the circulating adiponectin detected by ELISA increased from 9.97 ng/mL to 16.36 ng/mL in response to chronic-plus-binge alcohol (Fig.2.4.11A). Interestingly in A-Atg5 KO mice, the serum adiponectin was as high as 17.39 ng/mL at the basal level, and slightly decreased to 15.48 ng/mL after alcohol. Circulating adiponectin exists in different multimerized forms, and these complexes have different biochemical characteristics and activate different signal transduction pathways. Therefore, we checked multimerization of serum adiponectin protein in a non-heat denaturing and non-reducing condition (Waki et al. 2003). Consistently, we found increased high-molecular-weight (HMW) adiponectin in serum of WT mice after alcohol and in KO mice with or without alcohol treatment (Fig.2.4.11B). The mRNA level of liver *Adipor1* was increased 1-fold in response to chronic-plus-binge alcohol while *Adipor2* remained unchanged (Fig.2.4.11C). In A-Atg5 KO mice, the mRNA level of liver *Adopor1* was slightly higher and *Adipor2* was slightly lower than WT, and they were not changed after alcohol. This suggests that chronic-plus-binge alcohol treatment increased adipose adiponectin secretion possibly as an adaptive protection. The improved alcohol-induced liver injury in A-Atg5 KO mice was not due to a higher secretion of adiponectin.

Figure 2.4.11

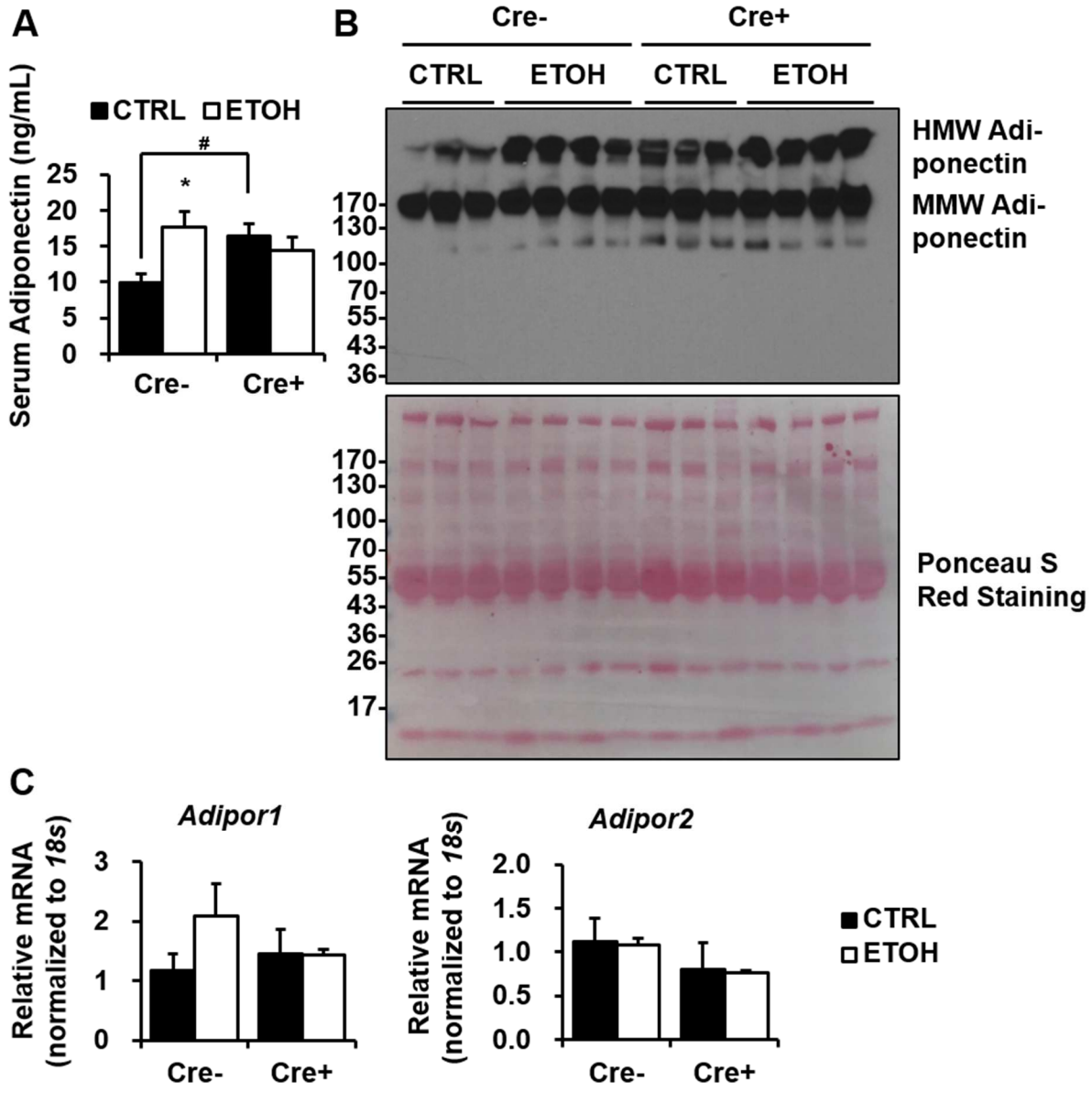

Figure 2.4.11 Serum adiponectin was increased in A-Atg5 KO mice and not further increased by chronic-plus-binge alcohol.

WT and A-Atg5 KO mice were treated with chronic-plus-binge alcohol, and serum and liver samples were collected 8h after binge. (A) Serum adiponectin level was measured by ELISA (n=6-8 mice per group). Data shown are mean±SEM, *p<0.05 (CTRL vs ETOH), #p<0.05 (Cre- vs Cre+) by Student's t test. (B) Serum was subjected to western blot in a non-heated, non-denatured condition. Ponceau S Red Staining was applied as the loading control. (C) Liver was subjected to mRNA extraction and qPCR (n=4-6 mice per group). Data shown are mean±SEM.

FGF21 is an anti-obesity, ant-diabetes hormone secreted by liver. It targets multiple organs including adipose tissue, and the role of FGF21 in ALD has been debatable (Zhao et al. 2015, Liu et al. 2016). We found that in WT mice, liver FGF21 had 72-fold increase in mRNA level and 2.5-fold increase in protein level in response to alcohol (Fig.2.4.12A-C). In addition, there was 12-fold increase in circulating FGF21 (Fig.2.4.11D). Interestingly, A-Atg5 KO mice had a higher basal level of liver FGF21 mRNA (5-fold) and circulating FGF21 level (2-fold) compared to WT mice. Liver FGF21 mRNA and protein levels were also increased after alcohol but by a smaller extent (20-fold and 1.8-fold, respectively) in A-Atg5 KO mice. Serum FGF21 level after alcohol was significantly lower in KO mice compared to WT. FGF21 binds to isoforms of FGF receptors facilitated by transmembrane protein β-Klotho/KLB. In liver we found there was no induction of mRNA level of *Klb* or *Fgfr4* in A-Atg5 KO mice, indicating the KO mice may not have enhanced affinity to FGF21.This suggests that lower liver injury in A-Atg5 mice was not due to a higher FGF21 level after alcohol. However, the high basal levels of FGF21 in A-Atg5 KO mice could prime the mice and make them less sensitive when they are exposed to alcohol.

Figure 2.4.12

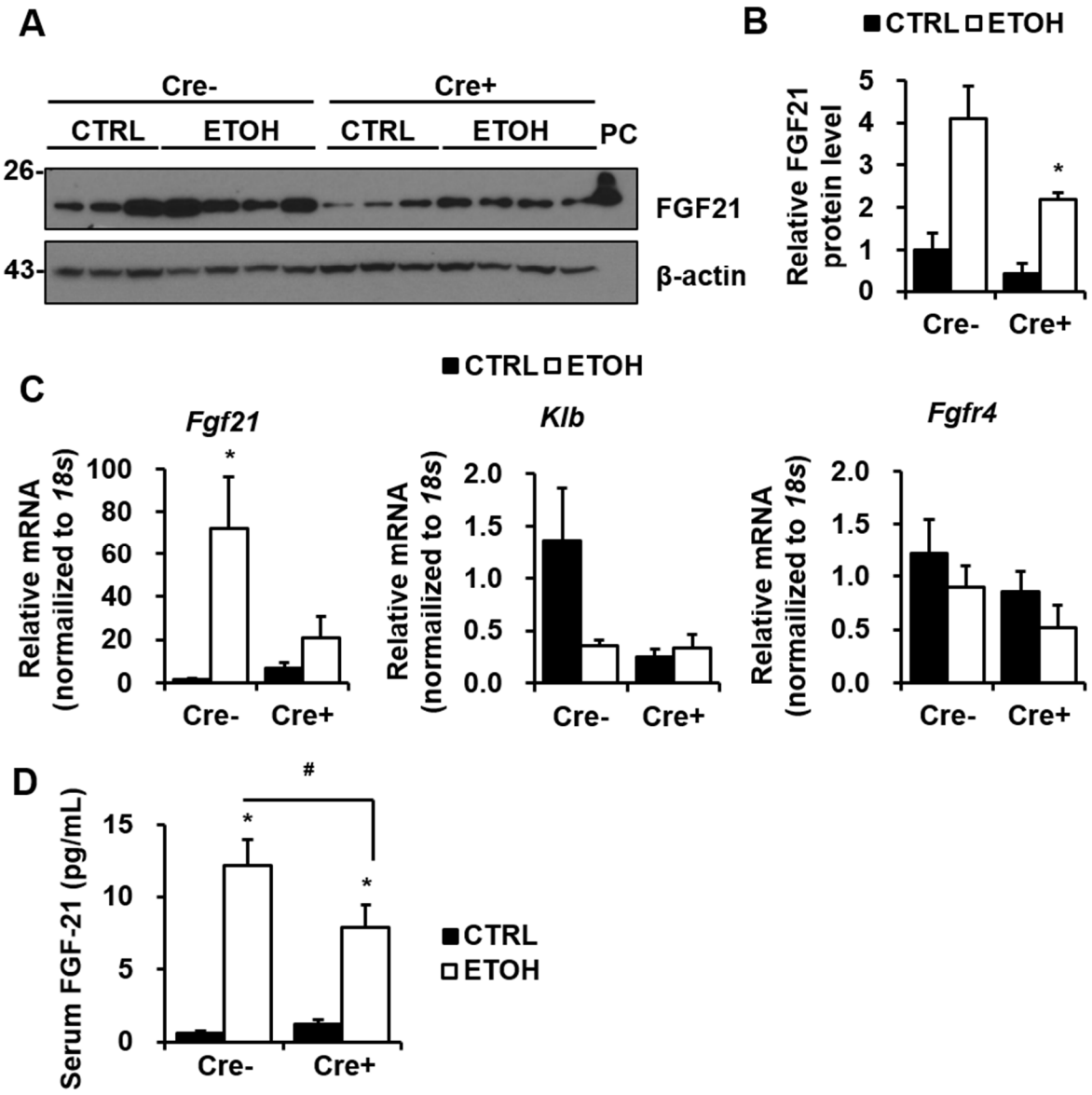

Figure 2.4.12 A-Atg5 KO mice had lower liver and serum FGF21 compared to WT after alcohol.

WT and A-Atg5 KO mice were treated with chronic-plus-binge alcohol, and serum and liver samples were collected 8h after binge. (A) Liver total protein was extracted and subjected to western blot. PC: FGF21 recombinant protein as positive control. β-actin was used as a loading control. (B) Densitometry of (A). (C) Liver was subjected to RNA extraction and qPCR (n=4-5 mice per group). (D) Serum FGF-21 level was measured by ELISA (n=8-13 mice per group). Data shown are mean±SEM, *p<0.05 (CTRL vs ETOH), #p<0.05 (Cre- vs Cre+) by One-way ANOVA.

2.4.7 Chronic-plus-binge Model Does Not Induce Adipose Inflammation in eWAT

Chronic alcohol exposure for 4 weeks in rats induces inflammation and macrophage infiltration into adipose tissue (Kang et al. 2007). We next investigated whether chronic-plus-binge alcohol induced inflammation in adipose tissue in mice. We examined several pro-inflammatory markers including *Tnfα*, *Il-6*, *F4/80* and *Ccl2*, and none of them increased by chronic-plus-binge alcohol. The expression levels of *Tnfα*, *Il-6*, *F4/80 and Ccl2* were also not different between WT and A-Atg5 KO mice (Fig.2.4.13A). Increased crown structures, i.e. dead adipocyte(s) surrounded by macrophages in adipose tissue, is another pathological marker of adipose inflammation. We found the presence of crown structures in eWAT of WT and A-Atg5 KO mice in both CTRL and ETOH group (Fig.2.4.13B), but the total number was too little in any condition (less than 5 per slide). This suggests that chronic-plus-binge alcohol likely induces very limited inflammation in eWAT.

Figure 2.4.13

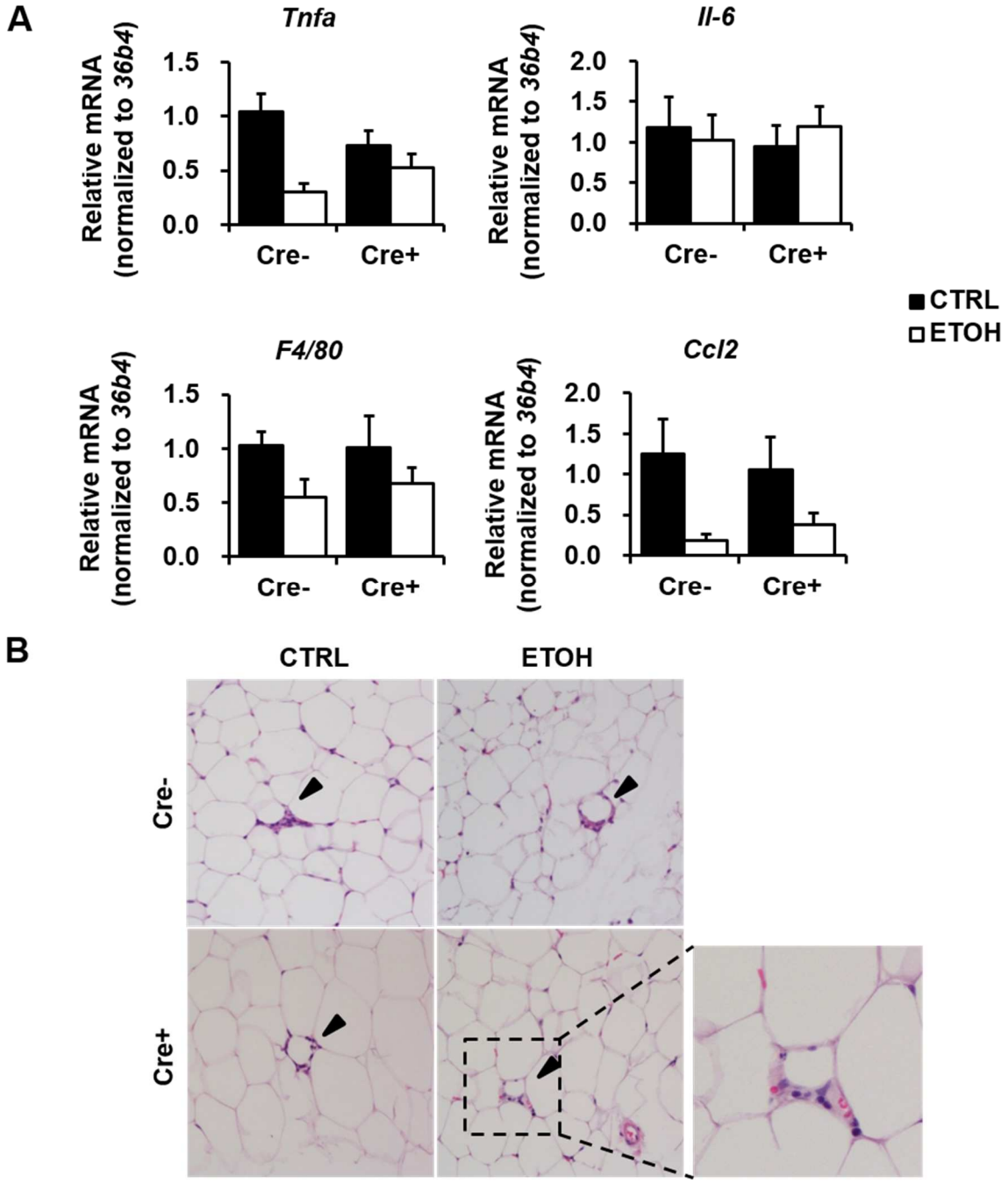

Figure 2.4.13 Inflammation and cell death was not increased in eWAT after chronic-plus-binge alcohol treatment in WT or Adipose-Atg5 deficient mice.

WT and A-Atg5 KO mice were treated with chronic-plus-binge alcohol, and eWAT samples were collected 8h after binge. (A) eWAT was subjected to mRNA extraction and qPCR (*n*=4-5 mice per group). Data shown are mean±SEM. (B) Representative images of crown structure (arrow head) in H&E staining (magnification=20x).

2.4.8 A-Atg5 KO Mice Are Not Affected in Isoproterenol-induced Lipolysis Ex Vivo

We continued to investigate the mechanisms of how A-Atg5 KO mice were protected from alcohol-induced adipose atrophy. We isolated eWAT and sWAT from WT and A-Atg5 KO mice and tested their response to isoproterenol, a beta adrenergic agonist that induces lipolysis. In WT mice, isoproterenol incubation increased free glycerol release by 1.5-fold in eWAT and by 2-fold in sWAT (Fig.2.4.14). The average free glycerol released in response to isoproterenol was slightly lower in A-Atg5 KO mice, but the induction fold was not significantly different from WT mice. This suggests that constitutional Atg5 deficiency in adipose tissue may not affect isoproterenol-induced lipolysis *ex vivo*.

Figure 2.4.14

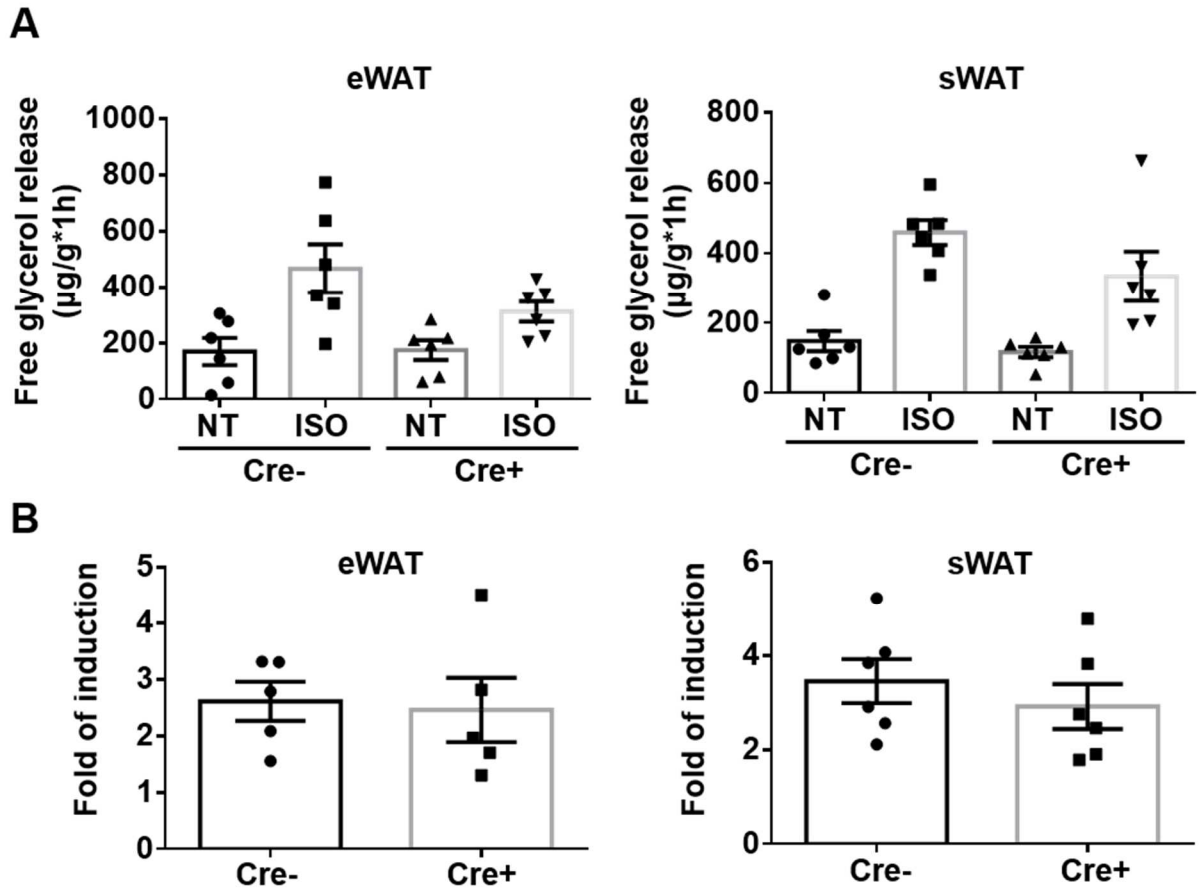

Figure 2.4.14 Adipose-Atg5 deficient mice were not affected in isoproterenol-induced lipolysis in fat *ex vivo*.
eWAT and sWAT were collected from WT and A-Atg5 KO mice and subjected to ex vivo lipolysis assay. Pieces of adipose tissues were incubated with or without isoproterenol (10 µM), and free glycerol released in the medium was measured after one hour and normalized to tissue weight. (A) Absolute values were presented and (B) folds of induction compared to non-challenged group were calculated. Data shown are mean±SEM.

2.5 Discussion

2.5.1 Chronic-plus-binge Alcohol Induces Mild Adipose Atrophy but Not Inflammation or Fibrosis

In our study, chronic-plus-binge alcohol treatment slightly decreased the mass of eWAT, sWAT, and rWAT while it significantly decreased BAT mass in Adipoq$^{f/f}$ Cre- mice. However, alcohol-induced reduction in adipose mass was more obvious and homogenous in 32-day chronic model and chronic-plus-binge model using C57BL/6J WT mice. Therefore, the mild adipose tissue atrophy we found in Cre- mice after alcohol is likely due to the mouse strain difference or different alcohol feeding models (see more detailed discussion below). Nevertheless, Cre- mice had more white adipocytes with smaller areas and brown adipocytes with decreased LD sizes in response to alcohol, therefore chronic-plus-binge alcohol may induce mild adipose atrophy in mice. In addition, we did not observe significant inflammation or cell death in adipose tissue after chronic-plus-binge alcohol treatment.

Several factors may contribute to the different response in adipose tissue to alcohol treatment. (1) Alcohol model: since length and route of alcohol administration affect the extent of liver injury, steatosis and survival rate (Brandon-Warner et al. 2012, Gao et al. 2017), it is possible that the extent of adipose atrophy, inflammation and fibrosis is also affected by different alcohol models. Decreased adipose mass and/or adipocyte size was observed in a 12-day chronic-plus-binge model in mice (Zhao et al. 2015), 4- to 24-week chronic feeding model in mice (Sun et al. 2012, Zhong et al. 2012, Crowell et al. 2016), and 4- to 12-week chronic feeding in rats (Kang and Nagy 2006, Zhang et al. 2015). So far there is no report of acute binge model inducing adipose atrophy, and in our hands the acute binge alone does not decrease adipose mass in WT mice (data not shown).

Increased adipose oxidative stress, inflammation and/or fibrosis was observed in a 25-day chronic feeding model in mice (Sebastian et al. 2011), and a 4-week chronic feeding model in rats (Kang et al. 2007, Tang et al. 2012). Interestingly in the 25-day feeding model, pro-inflammatory cytokines (TNF-α, IL-6, CCL2) and a fibrosis marker (CD11c) were transcriptionally upregulated at Day 25 but not at Day 18. Therefore, the extent of adipose atrophy and adipose injury (oxidative stress, inflammation) is likely to be time-dependent, and that might be why only mild adipose atrophy and no adipose injury was observed in our 10-day chronic-plus-binge model. In this chronic-plus-binge alcohol model, there is no severe lipodystrophy or increased pro-inflammatory cytokine secretion. However, a time-course experiment with longer exposure to alcohol and multiple binge may be very helpful. (2) Gene background: voluntary consumption of ethanol varies among different mouse strains (Belknap et al. 1993). Gao et al. reported that C57BL/6J mice were more sensitive to alcohol-induced mortality than C57BL6/N mice, they thus suggested that other background mice should be backcrossed into C57BL/6J background if genetically modified mice were used. In our lab, C57BL/6J WT mice seem to be more sensitive to alcohol-induced adipose atrophy than $Adipoq^{f/f}$ mice, though the $Adipoq^{f/f}$ mice were already backcrossed to C57BL/6J for more than 5 generations. It is not clear whether the different response is due to the different genetic background. We used littermates with similar gene background as WT control to minimize the influence from strains. (3) Gender: a study reported that female mice were more susceptible to chronic-plus-binge-induced liver injury, steatosis and adipose inflammation (Fulham and Mandrekar 2016). In our studies we used male mice for all *in vitro* assays. (4) Handling variation: though a standardized protocol has been established, it is still possible that the handing is different among researchers, resulting in various alcohol intake, binge-related mechanical injury, and stress in mice. Measurement of serum ethanol concentration will be helpful to make sure that comparable ethanol volume is conveyed to mice.

2.5.2 Constitutional *Atg5* Deletion Using *Adipoq-Cre* Does Not Lead to Adipose Tissue Atrophy at Least at Early Ages

A previous study reported that targeted *Atg7* deletion in adipose tissue using *aP2-Cre* resulted in lower body weight since 4-week old and drastically reduced WAT mass at age of 18 to 20 weeks (Zhang et al. 2009). Atg5 whole body KO embryos and neonatal pups had decreased subcutaneous fat cells (Baerga et al. 2009). Abundant evidence suggests that autophagy inhibition either genetically or pharmacologically impairs adipogenesis *in vitro*. Based on these findings, we hypothesized that *Atg5* KO in adipose tissue would result in impaired adipogenesis. However, we used 8- to 12-week male mice in our experiment, and found there was no significant difference in body weight or fat mass. We confirmed that there was partial *Atg5* deletion and autophagy inhibition in WAT and BAT. There was still LC3-II protein in WAT and BAT, so the recombination was not 100% and the remaining Atg5 might be enough for normal adipogenesis. We found that there was no difference in food intake or body weight change between WT and A-Atg5 KO mice, though physical activity and metabolic rate were not compared.

Except the possible difference in strains and animal facilities, a possible explanation of this phenomenon is that we used a different promoter *Adipoq-Cre* here, which is more specific than *aP2-Cre* in adipose tissue. So far there are no other studies using *Adipoq-Cre* to delete autophagy genes in adipose tissue. A study used *Ucp1-Cre* to delete Atg12 in brown and beige adipocytes did not report reduced adipose mass or impaired adipogenesis (Altshuler-Keylin et al. 2016). These KO mice had less adiposity expansion after 8-week high fat diet (HFD) challenge. It is possible that inhibiting autophagy using *Adipoq-Cre* or *Ucp1-Cre* is not potent enough to impair

adipogenesis, while these mice may have different response upon obegenic diet, diabetic diet or other metabolic stress. It is intriguing whether A-Atg5 KO mice will be less resistant to HFD-induced obesity or glucose challenge.

A study by Ghosh et al. used *aP2-Cre* to PI3K catalytic subunit type 3 (Pik3c3), which is a subunit of PI3K that regulates intermembrane trafficking and autophagy activity. The authors found reduced adiposity in middle-age (12-month-old) and old-age (24-month-old) mice but not young (4-month-old) mice. They found increased ER stress marker CEB/P homologous protein (CHOP) and autophagy substrate p62 in adipose tissue, but change in LC3 protein was not described, so it is unclear how potent autophagy inhibition was in this KO model, and whether any autophagy-independent mechanisms contribute to the KO phenotype. However, it indicates that change in adipose tissue phenotype might be age-dependent. Insufficient autophagy occurs during aging and contributes to aging diseases (Rubinsztein et al. 2011). It is possible that aging adipose tissue is more sensitive to stress. Currently there are few studies on autophagy in aging adipose tissue. Ghosh et al. found decreased autophagy activity in old SVFs (from 18 to 20 month-old mice) compared to young SVFs (from 4 to 6 month-old mice) associated with increased ER stress and inflammation (Ghosh et al. 2016). It will be interesting to determine whether there will be altered adiposity and different response to metabolic stress in older A-Atg5 KO mice.

Bif-1 is a positive mediator of autophagy by interacting with Beclin1 through UVRAG (Takahashi et al. 2007) and regulating Atg9 trafficking (Takahashi et al. 2011). *Bif-1* KO mice had dampened autophagy activity in adipose tissue, but, unlike *aP2-Cre Atg7* KO mice or *Atg5* whole body KO mice, they developed obesity with aging and gained more weight after HFD (Liu et al. 2016). These mice did not show impaired adipogenesis, and Bif-1 knockdown in 3T3-L1 cells did not inhibit preadipocyte differentiation. Therefore autophagy deficiency in adipose tissue may not

necessarily cause reduced atrophy, especially when other organs like liver and skeletal muscles were involved. To understand the role of autophagy in adipose tissue, using a tissue-specific KO model rather than whole body KO model will simplify the questions.

Notably, studies using *Adipoq-Cre* to delete *Raptor/mTOR* or *TSC1* exhibited reduced adipose tissue mass and body weight in mice at early ages (4 to 8 weeks) (Lee et al. 2016, Magdalon et al. 2016, Shan et al. 2016). Since the mTOR signaling pathway determines many biological processes like protein synthesis, it is likely that mice with mTOR modulation in adipose tissue have impaired adipogenesis in both autophagy-dependent and autophagy-independent mechanisms.

A limitation of using constitutional *Adipoq-Cre* in this study is that it may mix the impact of autophagy deficiency on adipogenesis at the early adipose development stage and the mature adipocytes. In ALD patients, usually adipogenesis is normal while the adipose tissue becomes unhealthy. To better determine the role of autophagy in mature adipocytes in ALD, an inducible KO model is preferable. One option is inducible Cre system which uses tamoxifen-dependent Cre recombinases (Feil et al. 2009). In this system, *Cre* sequence is fused with a tamoxifen-responsive estrogen receptor and retained in cytoplasm. When tamoxifen or 4-hydroxytamoxifen is administrated and binds to the estrogen receptor, CreER recombinase translocates into nucleus to work, and thus the timing of tissue-specific deletion is controlled. A $CreER^{T2}$ system was introduced to *Adipoq* gene and achieve 97-99% recombination in WAT and ~15% recombination in BAT (Sassmann et al. 2010). However, tamoxifen induces acute fat loss and de novo lipogenesis in mice (Ye et al. 2015). Researchers are still modifying this system to minimize the toxicity of tamoxifen. Therefore to better determine the effects of genetic loss of autophagy in mature adipocytes in response to alcohol exposure, inducible A-Atg5 KO mice should be used in the future studies.

2.5.3 Constitutional *Atg5* Deletion in Adipose Tissue Results in Increased Basal Levels of Adiponectin and FGF21 and Resistance to Alcohol-induced Liver Injury

In 2013, Dr. Gao's group established the chronic-plus-binge model containing 10-day alcohol feeding and an alcohol binge on the last day (Bertola et al. 2013). This model mimics human acute hepatitis and results in mild liver injury peaking at 9 h after binge and mild liver steatosis with no sign of fibrosis. Using this model, we found that the ALT and AST levels in A-Atg5 KO mice after chronic-plus-binge alcohol were much lower than WT in response to alcohol, therefore we concluded there was ameliorated alcohol-induced liver injury in A-Atg5 KO mice.

Though previous studies in Dr. Gao's group reported increased neutrophil infiltration and increased pro-inflammatory cytokines in liver and serum (Ki et al. 2010, Bertola et al. 2013, Xu et al. 2015, Li et al. 2017), we only found a slight increase in neutrophil infiltration, and no increase in liver mRNA levels of *Tnf-α*, *Il-1β*, or *Il-6*. We did observe increased mRNA levels of *Ccl2* and carbonylated proteins in WT mice after chronic-plus-binge alcohol. The extent of liver injury and inflammation seems to be milder than previously reported (Bertola et al. 2013), and this might be the reason we did not see a further decrease in these parameters in A-Atg5 KO mice. As discussed earlier, the milder liver injury and inflammation might be due to the differences in mouse strain, sex, handling, and animal facilities. Moreover, younger mice (8- to 12-week-old) tend to have less injury then older mice (>12-month-old) in this model (Ramirez et al. 2017).

To find the mediators responsible for A-Atg5-associated reduced liver injury after ethanol, we first checked adiponectin which is mainly secreted by adipocytes and can target liver. We found chronic-plus-binge alcohol increased serum total adiponectin level in WT mice, and A-Atg5 KO

mice had higher adiponectin at the basal level although it was not further increased after alcohol. The effect of alcohol on adiponectin level has been controversial in human studies (Sierksma et al. 2004, Beulens et al. 2007) and in rodent models. Four-week alcohol feeding in rats or mice decreased circulating adiponectin in some studies (Chen et al. 2007, Song et al. 2008). However, a 4-week alcohol feeding in rats showed increased circulating adiponectin (Pravdova et al. 2009). A study using chronic-plus-binge model reported elevated serum adiponectin after alcohol (Fulham and Mandrekar 2016). So far there is no study on the impact of acute binge on adiponectin, thus it is unclear whether acute binge is exerting an impact on adiponectin that masks the effect of chronic feeding. In addition, a 6-week high-fat liquid ethanol diet in mice recorded circulating adiponectin, and found it started decreasing since the 3rd week (Xu et al. 2003). It is possible that adiponectin is induced by alcohol as an adaptive response, and the decrease in adiponectin happens under chronic exposure and in a later time point. An intragastric infusion of overfeeding together with alcohol over 4 weeks in mice resulted in synergistic steatohepatitis with increased serum adiponectin, suggesting that adiponectin might be affected by obesity in additional to alcohol (Xu et al. 2011). Moreover, we found the levels of HMW adiponectin were correlated with total adiponectin level, and MMW adiponectin did not differ much between treatments or genotypes. Though it was suggested different oligomers of adiponectin might have different physiological functions (Tsao et al. 2003), several studies on obesity and type-2 diabetes in humans found the HMW adiponectin concentration is consistent with total adiponectin level and has no superiority in prediction value (Blüher et al. 2007, Bluher et al. 2007, Almeda-Valdes et al. 2010, Zhu et al. 2010). Adiponectin multimerization and HMW adiponectin in ALD is less studied. In our study, HMW level seemed to be associated well with total serum adiponectin, while MMW level did not change. Though the effect of alcohol on adiponectin is debatable, it is widely accepted that

adiponectin plays a protective role in ALD. Notably, A-Atg5 KO mice had increased total serum adiponectin and HMW adiponectin at basal levels although it was not increased after alcohol.

Another potential mediator of the adipose-liver axis is FGF21. As a protective metabolic regulator, FGF21 is mainly synthesized in liver and can target multiple tissues including adipose tissue. Consistent with previous studies (Zhao et al. 2015, Liu et al. 2016), we found chronic-plus-binge alcohol markedly increased liver and serum FGF21, which seems to be an adaptive response to liver injury. Interesting, in KO mice the basal levels of liver *Fgf21* mRNA and serum FGF21 were already increased significantly although the levels of FGF21 only increased mildly after exposure to alcohol compared with WT mice. In KO mice liver FGF21 protein was not paralleled with Fgf21mRNA, and it is possible that part of the FGF21 protein synthesized in liver was secreted to circulation. It should be noted that alcohol binge alone could induce serum FGF21 elevation peaking at 6 h and gradually restoring to normal in human and mice (Desai et al. 2017). Therefore the effect of alcohol on serum FGF21 in the chronic-plus-binge alcohol is a mixture of chronic exposure and acute binge. Our results suggest that it is possible that the increased basal levels of adiponectin and FGF21 in A-Atg5 KO mice may prime these mice to be more resistant to alcohol-induced liver injury. Future studies are needed to further dissect how basal levels of adiponectin and FGF21 would affect the liver response to alcohol.

2.5.4 Conclusions

In conclusion, chronic-plus-binge alcohol leads to decreased adipocyte/LD size in mice, which is associated with inhibited Akt/mTOR signaling and enhanced autophagy in adipose tissue. This model is not potent enough to induce significant adipose inflammation or fibrosis. *Atg5* deletion using *Adipoq-Cre* in adipose tissue does not significantly affect adipose mass in mice

possibly due to remaining autophagy-related proteins. However, these KO mice are more resistant to chronic-plus-binge alcohol-induced adipose atrophy. Moroever, A-Atg5 KO mice are more resistant to chronic-plus-binge alcohol-induced liver injury but not steatosis. The constitutional autophagy deficiency in adipose tissue leads to increased basal adipokine and hormone levels including increased circulating adiponectin and FGF21, which may enable these mice more resistant to alcohol-induced liver injury. The above findings are summarized in Fig.2.5.1.

Figure 2.5.1

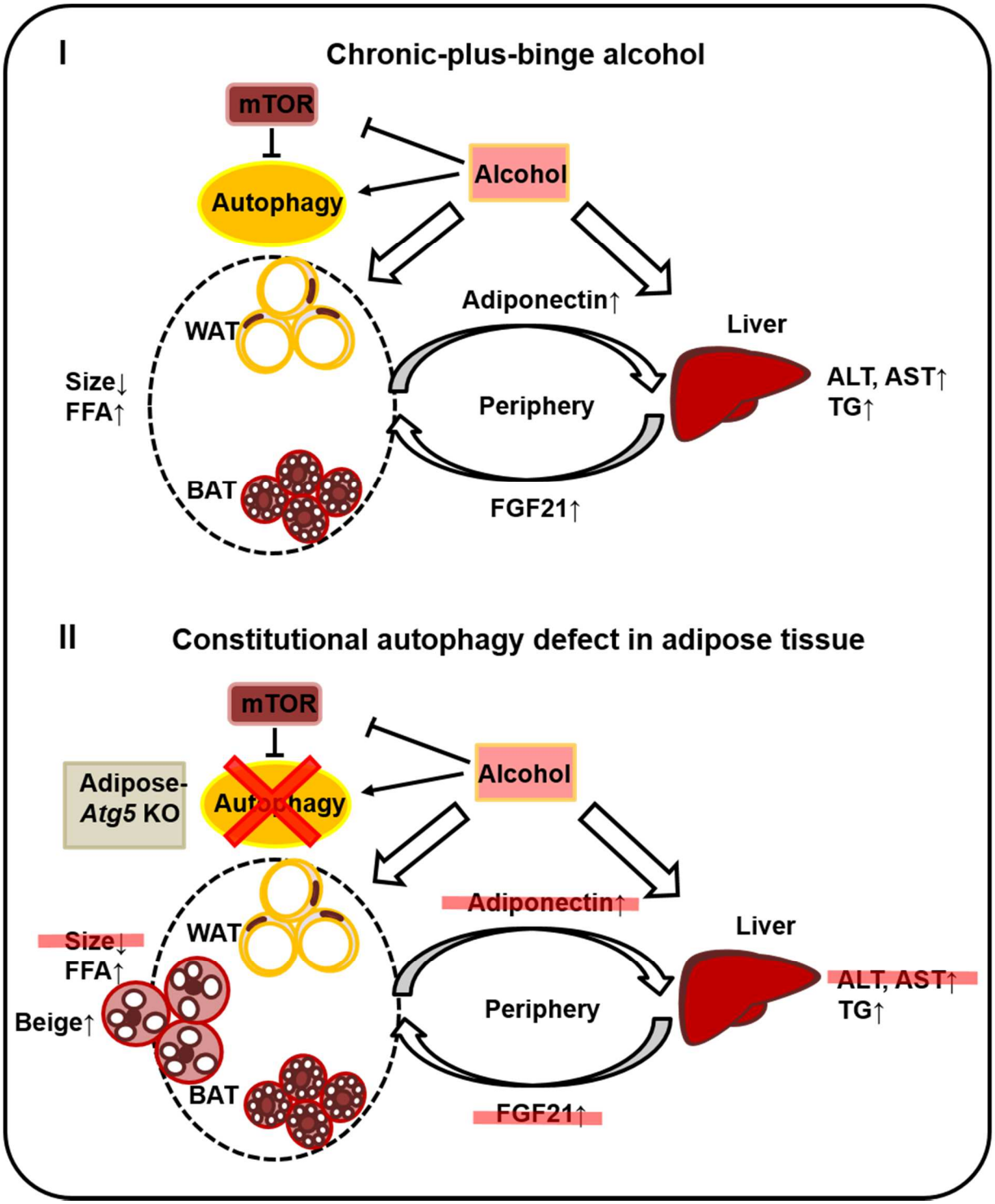
I
Chronic-plus-binge alcohol
mTOR
Autophagy
Alcohol
Adiponectin↑
WAT
Liver
Size↓
FFA↑
Periphery
ALT, AST↑
TG↑
BAT
FGF21↑
II
Constitutional autophagy defect in adipose tissue
mTOR
Adipose-
Atg5 KO
Autophagy
Alcohol
Adiponectin↑
WAT
Liver
Size↓
FFA↑
Periphery
ALT, AST↑
TG↑
Beige↑
BAT
FGF21↑

Figure 2.5.1 Scheme of the role of autophagy in chronic-plus-binge alcohol-induced adipose atrophy and liver injury.

Potential role of autophagy in alcohol-induced adipose atrophy and liver injury: I. Upon chronic-plus-binge alcohol, adipocyte autophagy is activated in mTOR-dependent and mTOR-independent pathways. Alcohol results in adipocyte atrophy and lipolysis, increased liver injury and steatosis, and increased serum adiponectin and FGF21 secretion as a protective response. II. In adipose-specific Atg5 KO mice, adipocyte autophagy is inhibited, and it subsequently increases beige adipocyte appearance and alleviates alcohol-induced adipose atrophy and liver injury, and decreases the secretion of protective mediators. WAT: white adipose tissue; BAT: brown adipose tissue; mTOR, mammalian target of rapamycin; Atg5, autophagy-related gene 5; KO, knockout; FFA: free fatty acid; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TG: triglyceride; FGF21: fibroblast growth factor 21

CHAPTER 3. EFFECT OF AUTOPHAGY AND ALCOHOL ON ADIPOCYTE DIFFERENTIATION *IN VITRO*

3.1 Abstract

We previously investigated the role of adipose autophagy in alcohol-induced adipose atrophy and liver injury/steatosis in Chapter 2. To further understand the underlying mechanisms, we used 3T3-L1 cell line, a type of preadipocyte that can be differentiated into adipocyte-like cells under adipogenic induction, as an *in vitro* model. We confirmed that 9-day incubation with adipogenic reagents led to a satisfying induction of adipocyte development by 3T3-L1 cells. We found during the adipogenesis of 3T3-L1 cells, there was increased mitochondrial protein but mitochondria showed diffuse staining pattern by tetramethylrhodamine methyl ester (TMRM) or MitoTracker, suggesting these mitochondria were undergoing abnormal remodeling. Electron microscopy studies also revealed significant accumulation of mitophagosome/mitochondrial spheroid like structures during the differentiation process. There was also increased autophagy degradation, and consecutive administration of lysosome inhibitor chloroquine (CQ) potently inhibited adipogenesis and changes in mitochondria. Constant administration of ethanol or acetaldehyde did not inhibit 3T3-L1 adipogenesis or changes of mitochondria morphology. In addition, short-term ethanol enhanced autophagy flux in differentiated 3T3-L1 adipocytes, and long-term ethanol exposure increased mitochondria protein levels. In summary, we found increased autophagic flux and dynamic mitochondrial remodeling during the *in vitro* differentiation of 3T3-L1 preadipocyte to adipocytes, which was not affected by ethanol exposure.

3.2 Introduction

3T3-L1 cells are mouse fibroblasts that tend to accumulate TG in the form of LDs. There are multiple LDs with the nucleus remaining centrally located, resembling brown adipocytes and differentiating white adipocytes. Established in 1974 (Green and Kehinde 1974), this cell line has been widely used to understand adipocyte physiology under controlled conditions. The exact recipes and protocols for inducing adipogenesis in 3T3-L1 cells vary, but the following reagents are generally included: (1) insulin, an adipogenic hormone that regulates a series of transcription factors including cAMP-response element binding protein (CREB) (Reusch et al. 2000); (2) thiazolidinedione like troglitazone and rosiglitazone, an insulin-sensitizer and PPAR-γ agonist (Tafuri 1996); (3) IBMX, a nonselective phosphodiesterase inhibitor and adenosine receptor antagonist that raises cAMP level and activates PKA (Elks and Manganiello 1985); (4) dexamethasone, a glucocorticoid that regulates a series of nuclear factors.

In our study, we applied 9-day incubation with a cocktail of the adipogenic reagents listed above to induce adipogenesis in 3T3-L1 cells. Using this *in vitro* model, we aimed to study the role of autophagy and mitochondrial changes in adipogenesis and the effect of alcohol on adipogenesis and adipocyte autophagy.

3.3 Materials and Methods

3T3-L1 Cell Culture and Adipogenesis Induction. 3T3-L1 cells were maintained at 37°C in a humidified 5% CO_2 atmosphere in DMEM (Hyclone) supplemented with 10% (v/v) FBS, penicillin and streptomycin (100 U/ml), and glutamine (100 μg/ml). To induce adipogenesis, 3T3-L1 cells were seeded and propagated to confluence. Forty-eight hours after 100% confluence, which was designated as Day 0, cells were incubated in differentiation medium [10 μg/ml insulin, 10μM troglitazone, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX)] for 3 days. At Day 3, cells were incubated in maintenance medium (5 μg/ml insulin, 10 μM troglitazone), and the maintenance medium was replaced every three days.

Oil Red O Staining. Oil Red O stock solution and Millipore water (v/v=6:4) was mixed well and filtered through 125 mm filter paper to make working solution before use. For cultured cells, cells were washed once with PBS, fixed with 4% PFA at RT for 1h, and washed with 60% 2-propanol twice for 5 min. Then the cells were counterstained with hematoxylin for 1 min and washed with PBS. For quantification, the Oil Red O content was extracted from cells using 100% 2-propanol, and the OD value was measured by spectrometer at λ=492 nm. 2-propanol was used as blank control.

Electron Microscopy. Cultured cells were seeded on Thermanox plastic coverslips (NUNC) in 35 mm dish and fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) followed by 1% OsO4. After dehydration, thin sections were stained with uranyl acetate and lead citrate. Digital images were captured using a JEM 1016CX electron microscope.

Protein Extraction and Western Blot. Cultured cells were scraped and culture medium was collected, followed by centrifugation at 200g x 5min, RT. The cell pellets were washed with PBS and centrifuged again at 200 g x 5 min, RT. Then the cell pellets were homogenized in ice-cold RIPA buffer. After incubation on ice for 30 min, samples were centrifuged at 12,000 g x 15 min at 4□. All protein lysates were supplemented with protease inhibitor cocktail. Supernatant was collected and protein concentration was determined by BCA assay. Samples were mixed with SDS loading buffer containing DTT and heated at 95 °C for 10 min unless otherwise indicated. Thirty micrograms of protein was separated by SDS polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Membranes were blocked in 5% milk in TBST at RT for 1 h, and then incubated with primary antibodies and secondary antibodies prepared in 5% milk in TBST. Signals were detected with SuperSignal West Pico Chemiluminescent Substrate and/or Immobilon Western HRP Substrate. Anti-LC3 antibody was generated as previously described (Ding et al. 2010). The following antibodies were used (Table 8). Densitometry was analyzed with Image J software. Band densities for the proteins of interest were normalized to β-actin unless otherwise indicated.

Table 8 Summary of antibody information in Chapter III

Antibody	Company	Catalog No.
β-actin	Sigma	A5441
GAPDH	Cell Signaling	2118
LAMP1	DSHB	1D4B

OXPHOS cocktail	Abcam	ab110413
p62/SQSTM1	Santa Cruz	sc-28359
VDAC	Calbiochem	AB10527

Fluorescence Microscopy. Cells were seeded on a 12-well plate. TMRM, LipidTOX, and MitoTracker Green dyes (Thermo Fisher) were used following the manufacturer's instructions, and images were taken in live cells. Fluorescence images were acquired under fluorescence microscope with MetaMorph software. For LipidTOX quantifications, cells with positive staining per field was counted for at least 4 fields in each group.

Statistical Analysis. Experimental data will be subjected to Student's *t*-test or one-way ANOVA analysis where appropriate. All error bars will be standard error.

3.4 Results

3.4.1 Autophagic Flux is Increased during Preadipocyte Adipogenesis

To determine autophagy status in adipogenesis of 3T3-L1 cells, we first established a 9-day 3T3-L1 *in vitro* adipogenesis model. In this model, 3T3-L1 cells were incubated in induction medium for 3 days (Day 0-Day 3) and then in maintenance medium for 6 days (Day 3-Day 9). Fibroblast-like cells gradually became round and accumulated LDs (Fig.3.4.1). The proportion of adipocyte-like cells and the LD size increased over time, and by Day 9 over half of the cells were differentiated into adipocyte-like cells. Cells undergoing induction were compared with cells incubated with normal medium with no adipogenic reagents, and lysosome inhibitor CQ was added for 6 h before harvesting to assess autophagic flux in each time point. We found in cells undergoing adipogenesis, the levels of LC3-II markedly decreased in a time-dependent manner, and CQ treatment further increased the levels of LC3-II protein especially in early adipogenesis (Fig.3.4.2A, B). Surprisingly, autophagy adaptor p62 was not affected during induction or by CQ. LAMP1 is an outer lysosomal membrane protein, interestingly the LAMP1 band shifted up during adipogenesis. Cells treated with an ATPase inhibitor BAF, also increased the levels of LC3-II protein in differentiating cells (data not shown). In differentiating 3T3-L1 cells that were co-treated with CQ for 9 days, there were a large amount of electron-dense vacuoles containing undegraded cellular components (Fig.3.4.2C). Taken together, this suggests that there was increased autophagic flux during adipogenesis of 3T3-L1 cells.

Figure 3.4.1

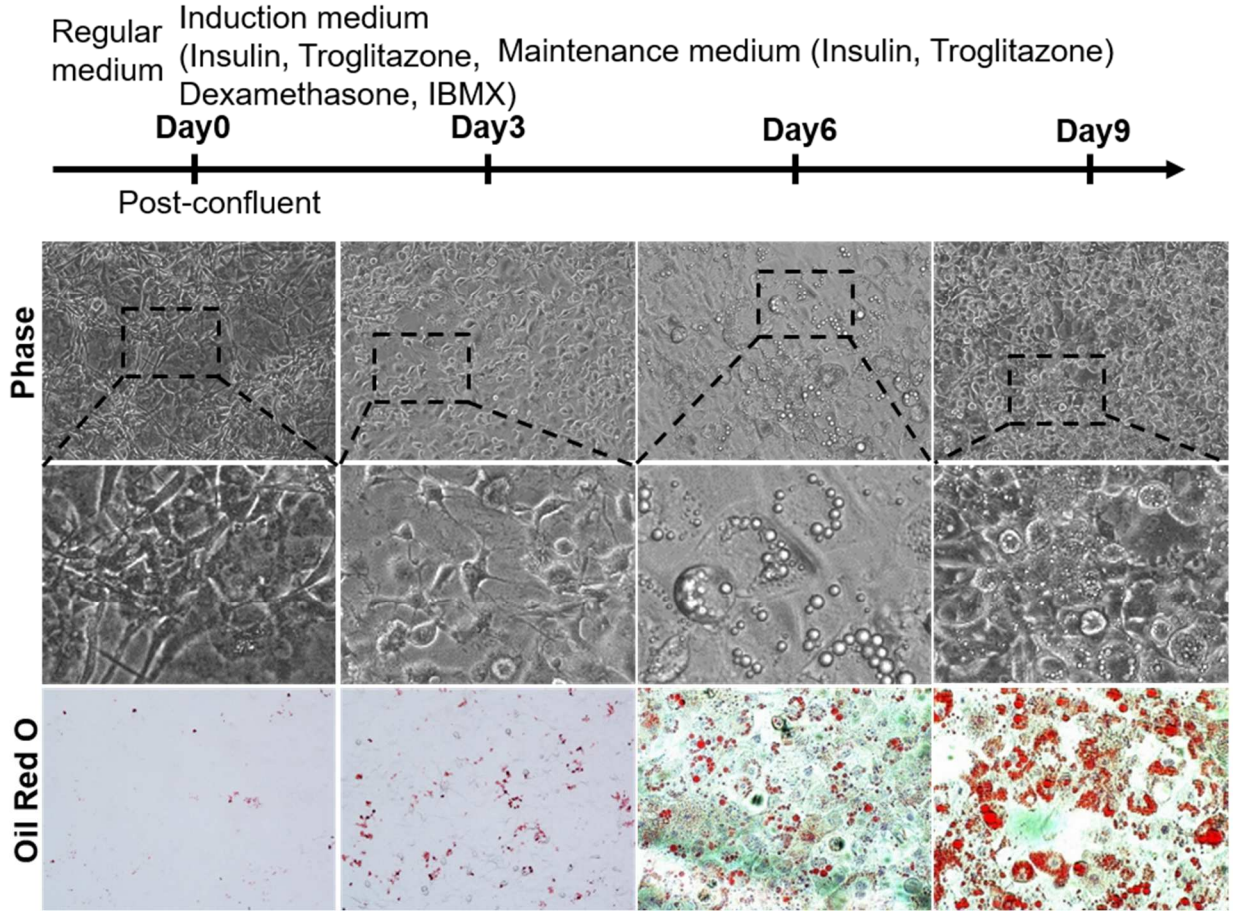

Fig 3.4.1 Schematic of adipogenic induction in 3T3-L1 preadipocytes.
Two days after 3T3-L1 cells were confluent, cells were incubated in induction medium for 3 days, and then in maintenance medium for 6 days. Representative phase image and Oil red O staining of differentiating cells on Day 0, 3, 6 and 9 (counted since induction medium was added) were shown (magnification=10x). Experiments have been repeated at least 3 times independently.

Figure 3.4.2

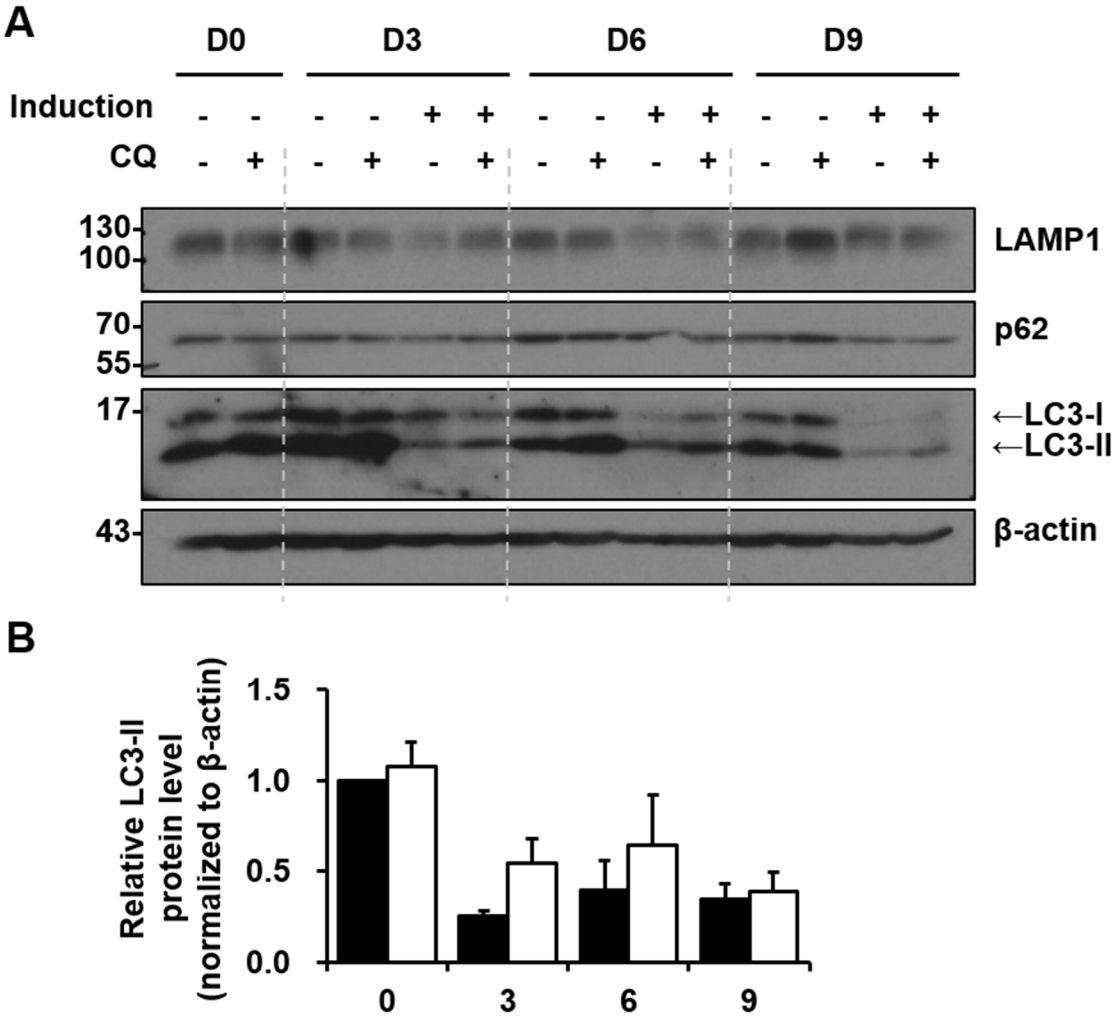

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Figure 3.4.2

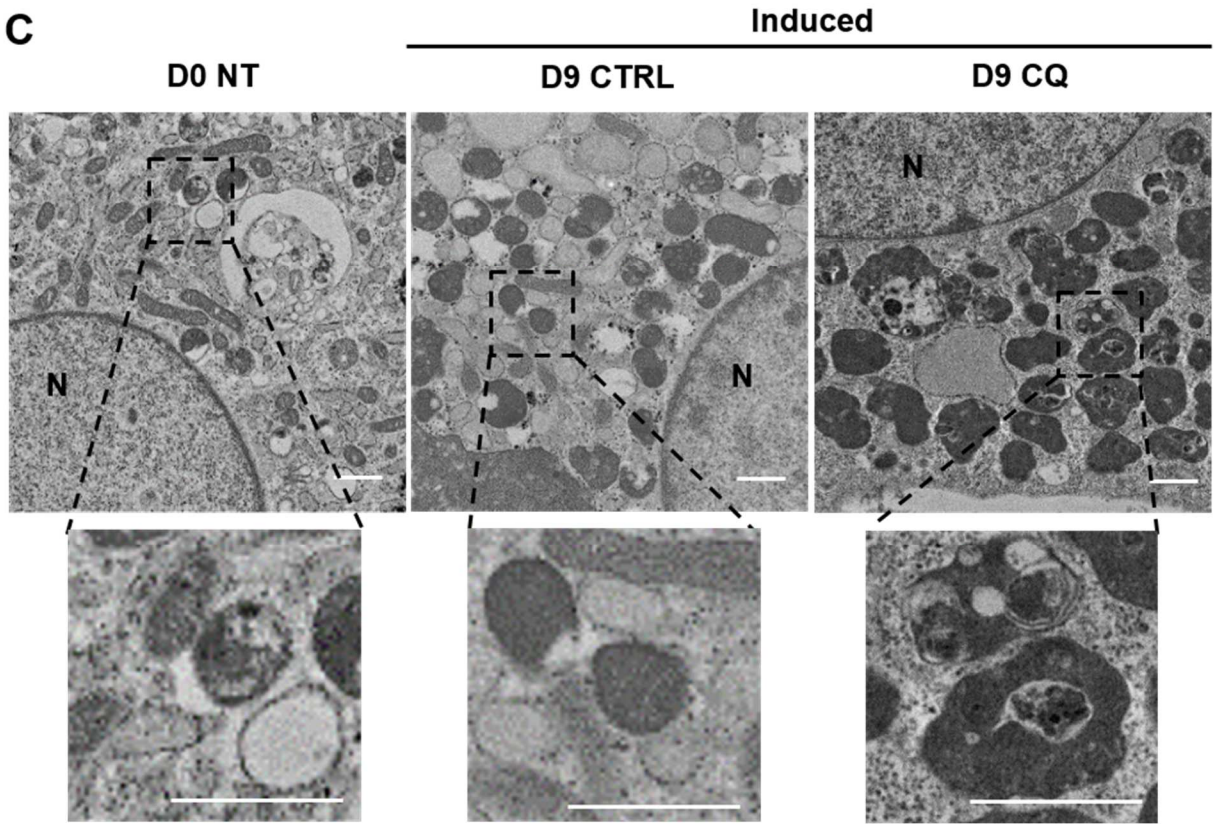


Figure 3.4.2. Adipogenesis in 3T3-L1 cells involved enhanced autophagy degradation. 3T3-L1 cells were induced for adipogenesis or maintained in regular medium for 9 days. (A) CQ (20 μM) was given 6 h before harvesting on Day 0, 3, 6 and 9, and cell lysate was subjected to western blot. β-actin was used as loading control. (B) Densitometry of (A) (*n*=4 individual experiments). (C) Representative EM images of non-treated (NT) cells on Day 0, differentiated (CTRL) cells on Day 9, and differentiated cells cotreated with CQ (20 μM) daily on Day 9. N: nucleus. Scale bar: 1 μm.

3.4.2 Dynamic Mitochondrial Remodeling During 3T3-L1 Preadipocyte Adipogenesis

Mature white adipocytes are characterized by scarce cytoplasm and few mitochondria. It was proposed that autophagy is essential for the drastic remodeling of preadipocytes during differentiation at least by removing abundant mitochondria and cytoplasmic components (Baerga et al. 2009, Zhang et al. 2009). On the other hand, increased mitochondrial biogenesis was observed in 3T3-L1 preadipocyte adipogenesis (Wilson-Fritch et al. 2003). To determine the change of mitochondria content and function during adipogenesis, we examined levels of several mitochondrial proteins in differentiating 3T3-L1 cells. Cells undergoing induction had increased levels of oxidative phosphorylation (OXPHOS) complex I, II, III and V residing on inner mitochondria membrane (Fig.3.4.3A) over the time-course of 3T3-L1 differentiation. Moroever, there was increased voltage-dependent anion channel (VDAC) protein which localizes on the outer mitochondria membrane, which reached the highest levels in Day 9. CQ treatment for 6 h did not increase OXPHOS complexes or VDAC proteins. This suggests that there were increased mitochondria proteins in differentiating 3T3-L1 cells, and lysosomal degradation seems have little impact on the accumulated mitochondrial proteins. We found abundant elongated mitochondria with clear cristae structures in non-differentiated 3T3-L1 cells on Day 0 using EM. Surprisingly, much fewer numbers of mitochondria with clear cristae structures were found in 3T3-L1 cells that were cultured with the induction medium for 9 days (Fig.3.4.3B). Interestingly, we found increased number of round, electron-dense vesicles on Day 9. To find out whether there were more functioning mitochondria or only accumulation of dysfunctional morphology-remodeled mitochondria during adipogenesis, we performed TMRM and MitoTracker staining in differentiating cells. TMRM dye is cationic and stains mitochondria due to the mitochondrial

membrane potential and deceased mitochondrial membrane potential leads to loss of TMRM staining. MitoTracker is also a cationic fluorescence dye but it is usually sequestered in mitochondria due to its covalent binding with mitochondrial thiol-reactive chloromethyl moiety proteins. On Day 0, sharp elongated TMRM-positive mitochondria were observed in undifferentiated 3T3-L1 cells. Intriguingly, both the TMRM and MitoTracker signals were much stronger in adipocyte-like cells (with round shape and LD accumulation) than fibroblast-like cells, though the TMRM and MitoTracker signals were more diffused (Fig.3.4.3C). The diffuse mitochondria staining pattern by TMRM and MitoTracker in differentiated cells are somewhat associated with the markedly increased electro-densed structures identified by EM studies. Notably, CQ treatment during adipogenesis preserved the sharp elongated mitochondria structure just like cells incubated in regular medium, suggesting inhibition of lysosomal functions have little effects on mitochondrial remodeling during the 3T3-L1 differentiation. Taken together, there was increased autophagic flux and dynamic mitochondrial remodeling during adipogenesis.

Figure 3.4.3

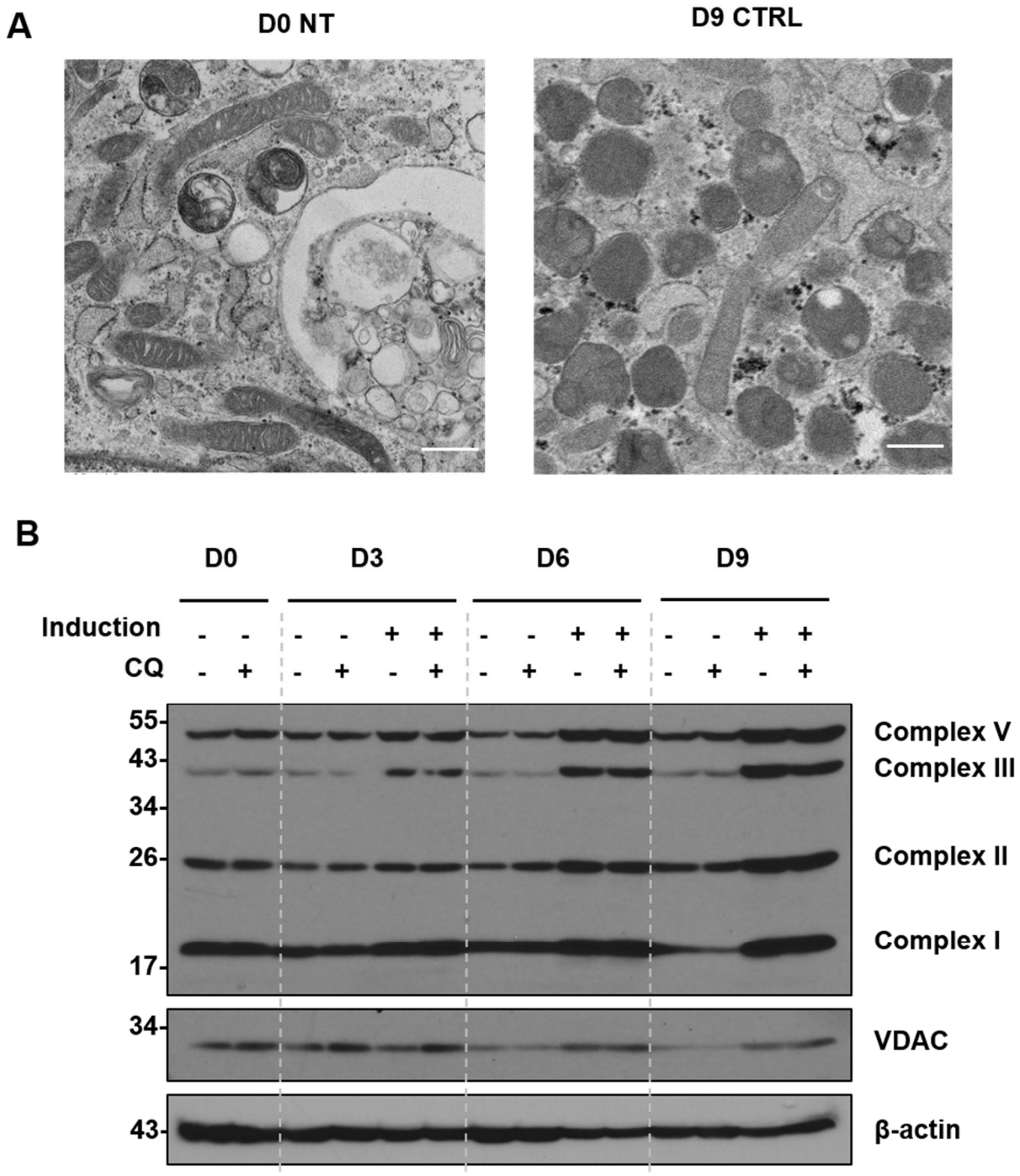

A
D0 NT
D9 CTRL
B
D0
D3
D6
D9
Induction
CQ
55
43
34
26
17
34
43
Complex V
Complex III
Complex II
Complex I
VDAC
β-actin

Figure 3.4.3 (Cont.)

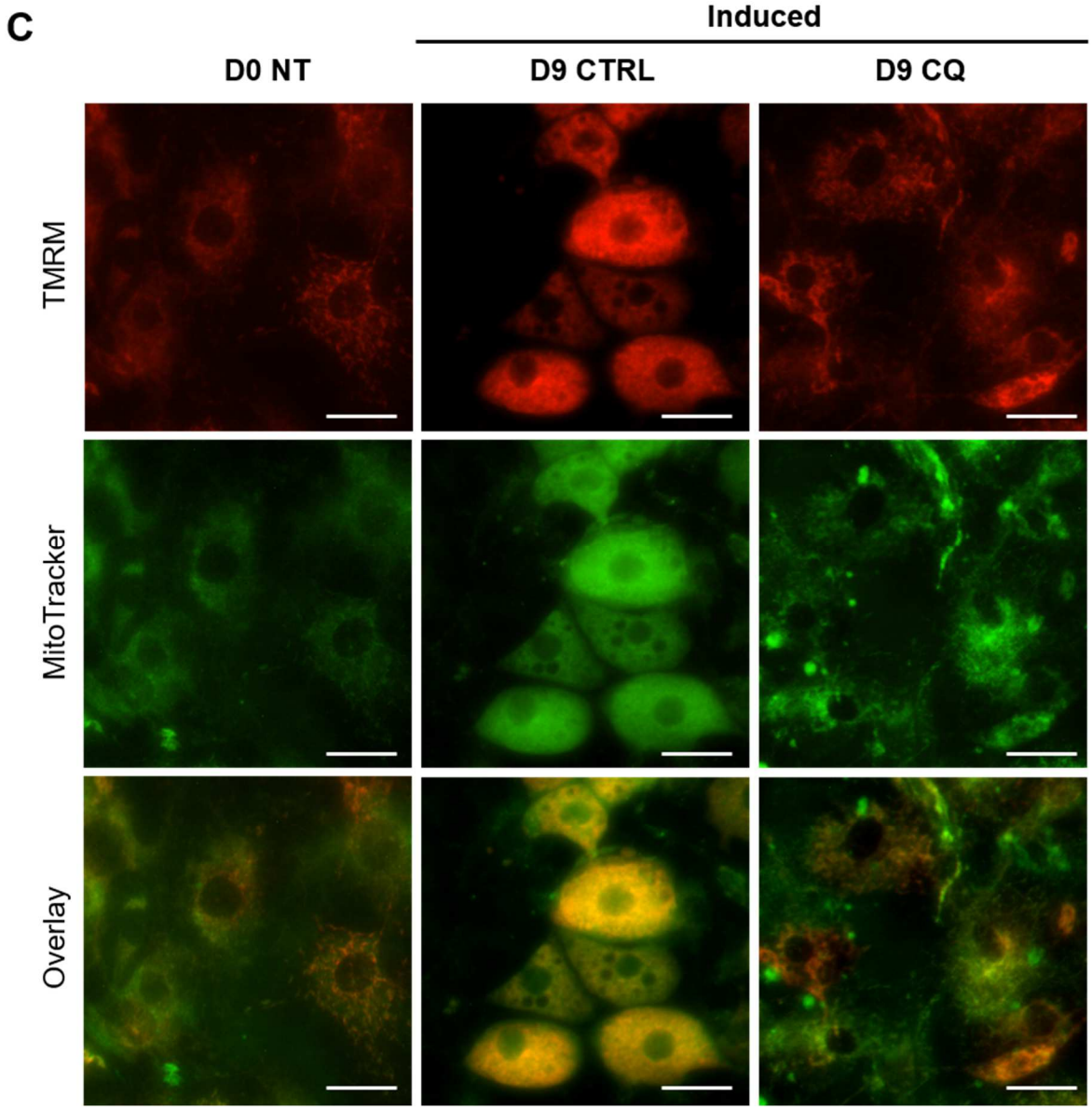


Figure 3.4.3. Adipogenesis in 3T3-L1 cells involved increasing mitochondria proteins and remodeling.

3T3-L1 cells were induced for adipogenesis for 9 days. (A) Cells undergoing induction or incubated with normal culture medium were treated with or without CQ (20 μM) for 6 h before harvested on Day 0, 3, 6 and 9, and cell lysate was subjected to western blot. β-actin was used as loading control. (B) Representative EM image of non-treated cells on Day 0 and differentiating cells on Day 9. N: nucleus. Scale bar: 500 nm. (C) Representative fluorescent images of non-treated cells on Day 0, differentiated cells on Day 9, and differentiating cells treated with CQ (20 μM) daily on Day 9 are shown. Live cells were subjected to TMRM and MitoTracker Green costaining. Scale bar: 20 μm.

3.4.3 Ethanol Does Not Inhibit 3T3-L1 Preadipocyte Adipogenesis

To determine the effect of ethanol on 3T3-L1 adipogenesis, 3T3-L1 cells undergoing differentiation were treated with 100 mM of ethanol, 100 μM of acetaldehyde or 20 μM of CQ daily for 9 days. Adipogenic induction increased cells containing LDs at Day 9 as indicated by Oil red O staining (Fig.3.4.4A, C) and LipidTOX staining (Fig.3.4.4B, D), which was potently inhibited by constant CQ treatment. Ethanol or acetaldehyde cotreatment did not significantly decrease cells with positive Oil red O staining or LipidTOX staining compared with control. This suggests that ethanol or acetaldehyde does not affect the adipogenesis rate in 3T3-L1 preadipocytes.

Figure 3.4.4

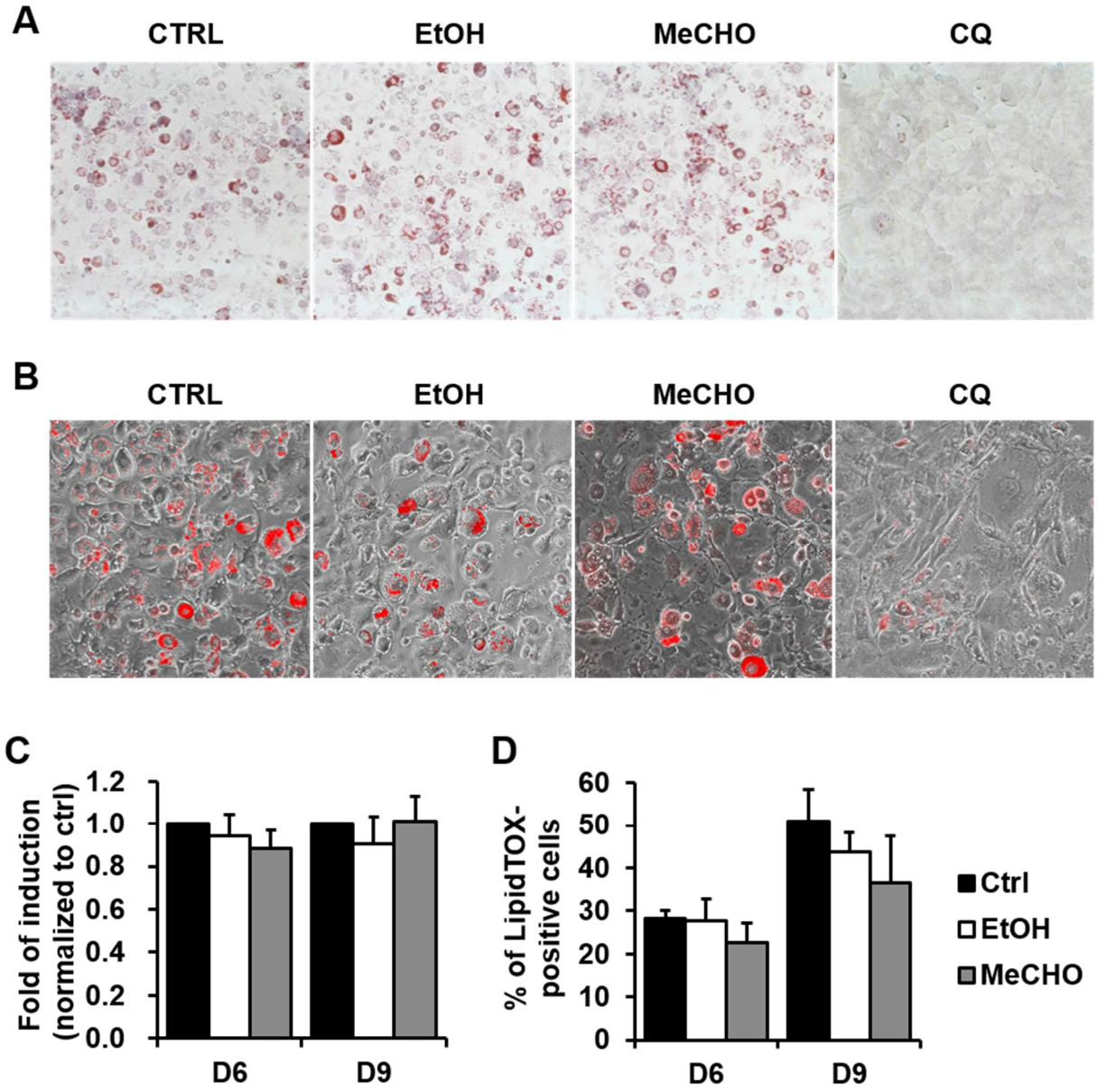


Figure 3.4.4. Ethanol and acetaldehyde did not inhibit adipogenesis in 3T3-L1 cells. 3T3-L1 cells were induced for adipogenesis and cotreated with ethanol (ETOH) (100 mM), acetaldehyde (MeCHO) (100 µM) or CQ (20 µM) daily for 9 days. (A) Representative images of Oil Red O staining of cells at Day 9 (magnification=20x). (B) Representative images of LipidTOX staining of cells at Day 9 (overlaid with Phase image, magnification=20x). (C) Quantification of Oil Red O staining at Day 6 and Day 9 (*n*=3-4 individual experiments). (D) Quantification of LipidTOX staining at Day 6 and Day 9 (*n*=3 individual experiments). Data shown are mean±SEM.

3.4.4 Ethanol Increases Autophagic Flux in Differentiated 3T3-L1 Adipocytes

The effect of ethanol on autophagy in 3T3-L1 adipocytes has not been studied. To test whether ethanol or acetaldehyde affects autophagy flux, 3T3-L1 cells undergoing 9-day differentiation were treated with ethanol or acetaldehyde for 24 h in the presence or absence of CQ. Ethanol alone increased LC3-II protein levels (1.5-fold), and CQ alone increased LC3-II protein levels to (1.7-fold). Ethanol plus CQ further increased LC3-II protein (2.1-fold) compared with ethanol or CQ alone, while acetaldehyde plus CQ did not further increase levels of LC3-II protein compared with acetaldehyde or CQ alone (Fig.3.4.5). The levels of p62 protein were not significantly affected by any of the treatments. The effect of prolonged treatment of ethanol or acetaldehyde (daily for 3 days) had similar effects on p62 and LC3 protein (data not shown). This suggests that ethanol treatment but not acetaldehyde may increase autophagic flux in 3T3-L1 cells.

Figure 3.4.5

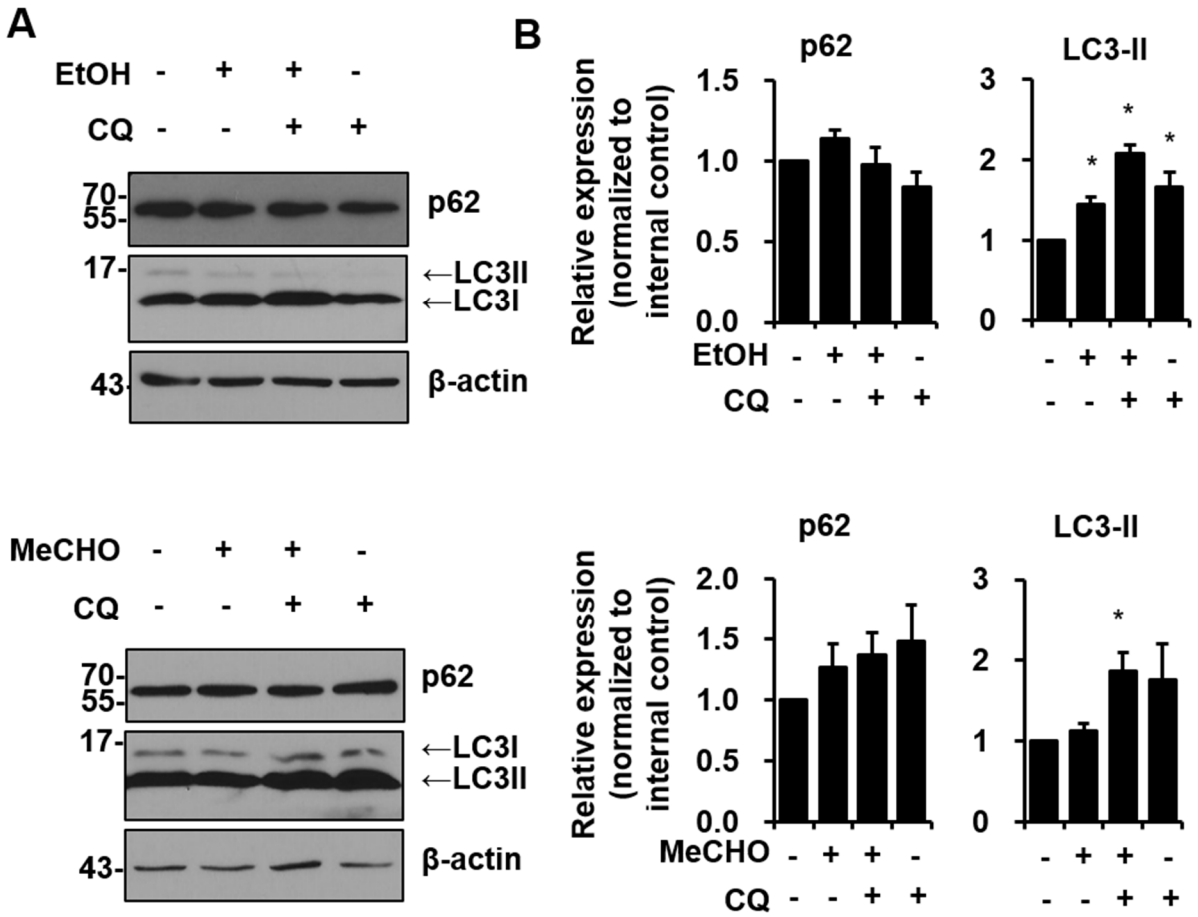


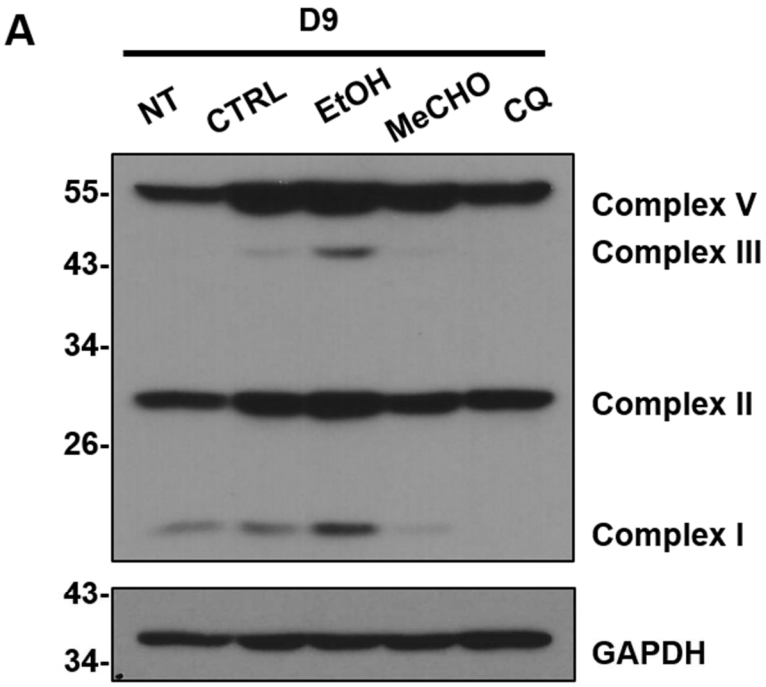
Figure 3.4.5. Ethanol but not acetaldehyde induced LC3II turnover in differentiated 3T3-L1 cells.

3T3-L1 cells were induced for adipogenesis for 9 days. On Day 9, cells were treated with EtOH (100 mM) or MeCHO (100 μM) for 24 h and/or treated with CQ (20 μM) for 6 h before cell lysate was collected. Cells were subjected to protein extraction and western blot. (A) Representative western blot of p62 and LC3. β-actin was used as loading control. (B) Densitometry of 4 individual experiments. Data shown are mean±SEM. *$p<0.05$ by Student's *t* test.

3.4.5 Ethanol Increases Mitochondrial Proteins in Differentiating 3T3-L1 Cells

The effect of alcohol on mitochondria content and function in 3T3-L1 during adipogenesis has not been well characterized. To determine the effect of alcohol on mitochondrial proteins, adipogenesis was induced in 3T3-L1 cells concomittant with ethanol (100 nM) acetaldehyde (200 μM) or CQ (20 μM) treatment for 9 days. Consistently, the levels of OXPHOS complex I, II, III and V protein l all increased in cells undergoing adipogenesis compared with cells incubated with normal medium, and CQ treatment inhibited the increase of OXPHOS complexes (Fig.3.4.6A). Long-term ethanol treatment further increased the protein levels of all complexes, while acetaldehyde treatment did not. Similar to cells undergoing normal adipogenesis, EM studies showed that cells treated with ethanol or acetaldehyde had only a few mitochondria with clear cristae while they had an increased number of electron-dense vacuoles with undegraded cellular components (Fig.3.4.6B). To find out whether the morphology or the membrane potential of mitochondria were affected by alcohol, 3T3-L1 cells were stained with TMRM and MitoTracker Green. 3T3-L1 cells treated with ethanol or acetaldehyde had similar enhanced TMRM and MitoTracker signals in adipocyte-like cells compared with fibroblast-like cells (Fig.3.4.6C). In these groups, the TMRM signal was diffuse and not always colocalized with MitoTracker signal. These data suggest that ethanol but not its metabolite acetaldehyde further increases mitochondrial proteins but it seems that neither ethanol nor acetaldehyde affects the mitochondrial remodeling during the adipocyte differentiation.

Figure 3.4.6


A
D9
NT
CTRL
EtOH
MeCHO
CQ
55-
43-
34-
26-
Complex V
Complex III
Complex II
Complex I
43-
34-
GAPDH

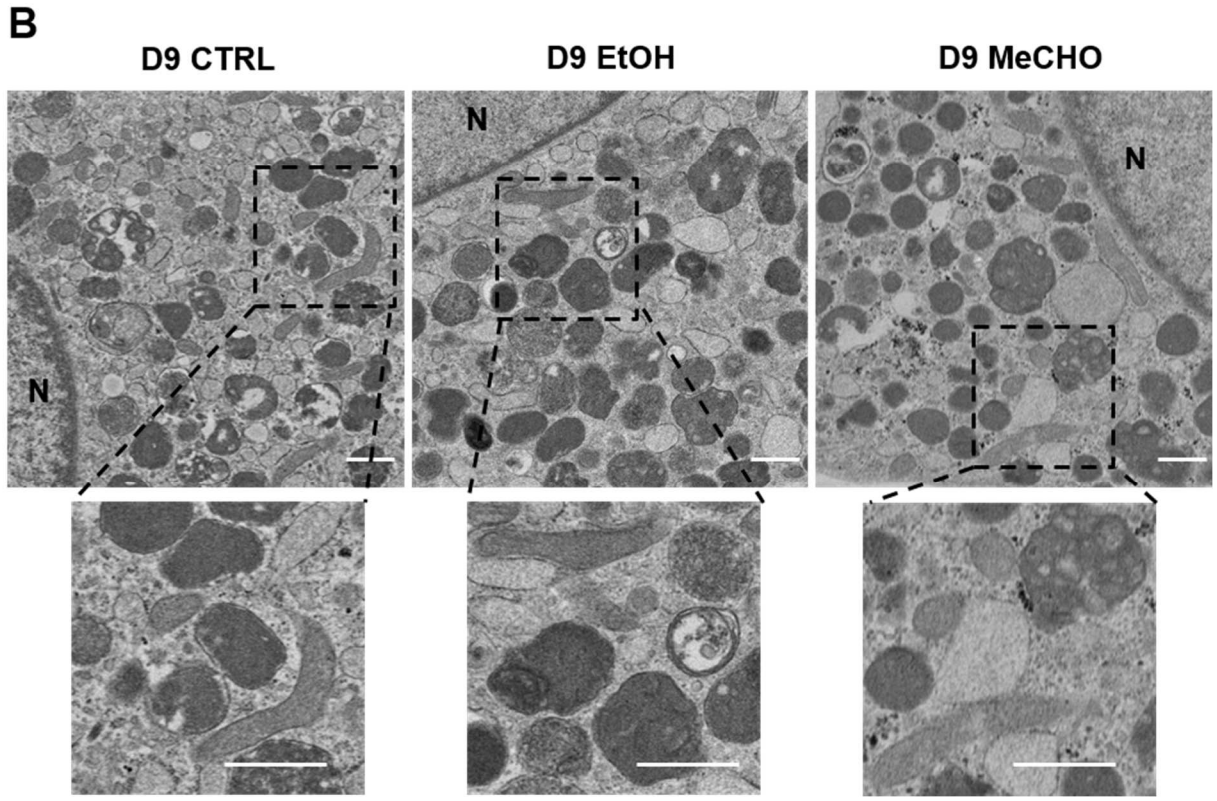

B
D9 CTRL
D9 EtOH
D9 MeCHO
N
N
N

Figure 3.4.6 (Cont.)

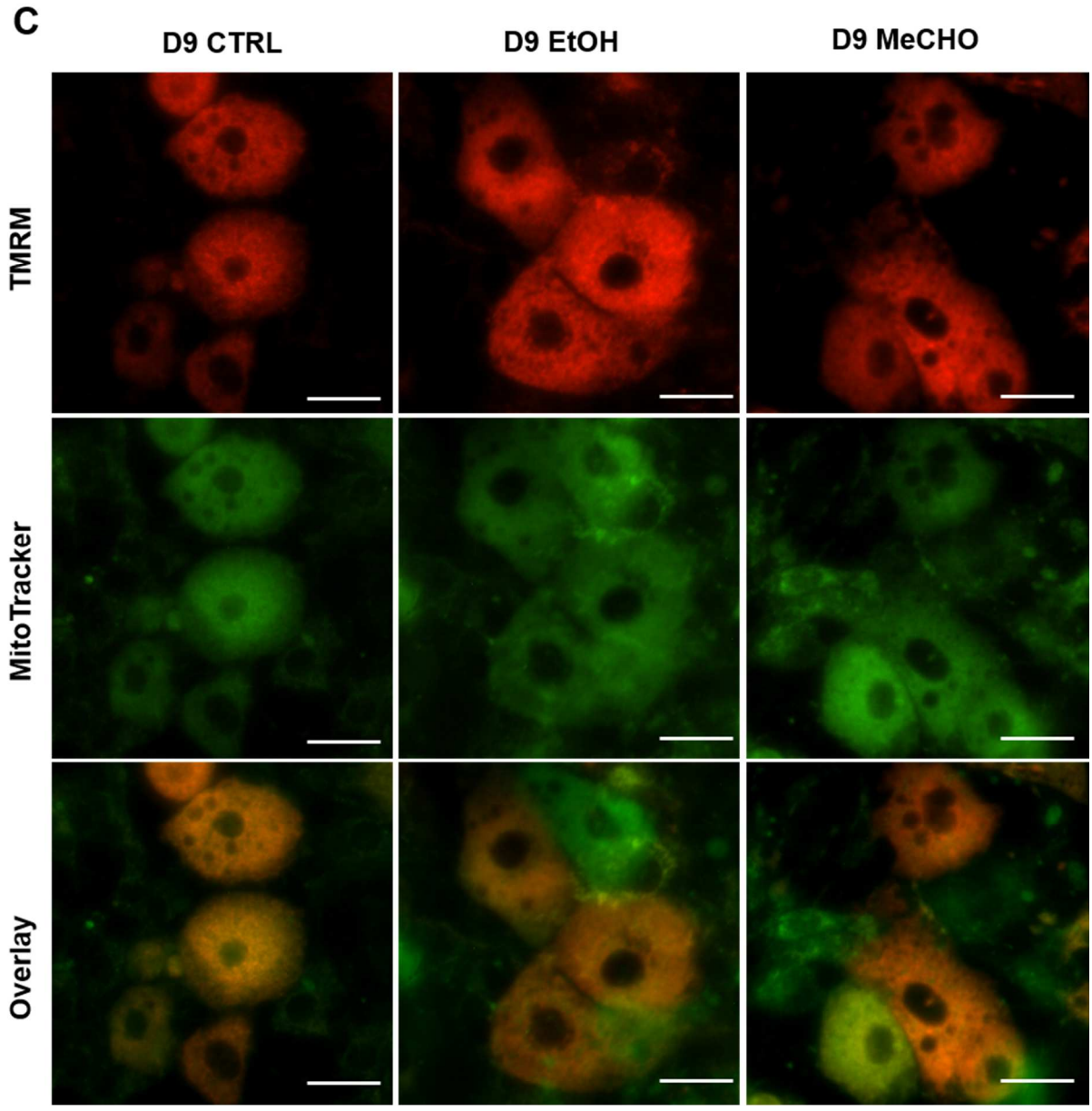


Figure 3.4.6. Ethanol but not acetaldehyde increased mitochondria protein in 3T3-L1 cells.

3T3-L1 cells were incubated with normal medium (NT) or undergoing adipogenesis with no other treatment (CTRL), EtOH (100mM), MeCHO (200μM), or CQ (20μM) cotreatment daily for 9 days. (A) Representative western blot of OXPHOS complexes. Cell lysate was collected on Day 9. GAPDH was used as loading control. (B) Representative EM images of differentiating cells with no other treatment, EtOH and MeCHO. N: nucleus. Scale bar: 1 μm. (C) Representative fluorescent images of differentiating cells with no other treatment, EtOH and MeCHO. Live cells were subjected to TMRM and MitoTracker Green costaining. Scale bar: 20 μm.

3.5 Discussion

3.5.1 Increased Autophagic Flux and Mitochondrial Remodeling in Adipogenesis of 3T3-L1 Preadipocytes

In this study, we found that during adipogenesis, there was increased autophagic flux and levels of mitochondria OXPHOS and VDAC proteins. In EM studies, fewer elongated mitochondria with clear cristae structure were observed in mature adipocytes, while increased electron-dense vacuoles with undegraded cellular components appeared. While the differentiated adipocytes still have TMRM and MitoTracker staining, they showed striking diffuse pattern, suggesting possible altered mitochondrial structures and functions. It is possible that the electron-dense vacuoles we saw might have derived from mitochondria and have contact with lysososomes/autolysosomes. This is supported by the observation that consecutive CQ treatment further increased the undegraded cellular components in these spheroids. Moreover, it is also possible that mitochondrial pH may be altered and become more acidic during the differentiation process of preadipocytes. This notion is supported by the observation that CQ-treated cells have the normal mitochondrial TMRM and MitoTracker staining, which is likely due to the neutralization of mitochondria pH by CQ. Future studies to co-stain cells with MitoTracker and LysoTracker in 3T3-L1 cells undergo differentiation will be helpful. We are also in the process of cloning a mitochondrial pH sensor which may also help us to test the mitochondrial pH hypothesis in the future. The lysosomal/autolysosomal removal of cellular components might be the key step or mitochondria remodeling. A proteomic analysis comprehensively studied mitochondria proteins in differentiating 3T3-L1 cells through day 0 to 18 (Newton et al. 2011). This study finds that during adipogenesis, 3T3-L1 adipocytes enter a “metabolic-overdrive” where TCA cycle and FA oxidation are enhanced. Interestingly, it was reported that pharmacological inhibition of autophagy

in early but not in later stages prevented 3T3-L1 adipogenesis by affecting mitochondria remodeling (Skop et al. 2014). Unfortunately, 3T3-L1 cells are not an ideal tool for genetic modulation to test the role of autophagy. Future studies to use COX8-GFP-mCherry system will help to better understand the dynamic process of mitochondria turnover. Costaining of mitochondria protein with LC3 or lysosome marker will be helpful to know whether the turnover is autophagosome/lysosome-dependent.

3.5.2 Alcohol Does Not Impair Autophagy or Mitochondria Turnover in 3T3-L1 Preadipocytes

We found that short-term ethanol treatment induced autophagy, and long-term ethanol treatment enhanced mitochondria proteins in 3T3-L1 cells, both favoring 3T3-L1 adipogenesis. However, constant alcohol or acetaldehyde treatment did not affect LD accumulation or mitochondria change during adipogenesis Alcohol is mainly metabolized by ALDH and CYP2E1in liver and adipocytes. However alcohol metabolism in 3T3-L1 cells is not well understood. It was reported that exogenous CYP2E1 expression contributed to alcohol-induced oxidative stress and impaired adiponectin secretion in 3T3-L1 cells (Tang et al. 2012). Another study reported that ALDH2 activation promoted 3T3-L1 adipogenesis (Yu et al. 2016). It is not clear why in our hands, only alcohol but not acetaldehyde posed effects on autophagy and changes of mitochondria OXPHOS proteins. Since there are only few studies of ethanol in 3T3-L1 cells, the dose of ethanol and acetaldehyde we applied was based on the common dose used in other cell types like hepatocytes. Shorter time-course experiments (e.g. 6 h) and dose-response experiments are needed to fully characterized the effect of ethanol or acetaldehyde in 3T3-L1 cells. Enzyme expression and activity of CYP2E1, ADH, ALDH and ALDH2 should be also examined in the future

3.5.3 Conclusions

In conclusion, 3T3-L1 preadipocyte adipogenesis involves enhanced autophagy and mitochondria remodeling. Autophagy is critical to the completion of mitochondria turnover. Ethanol does not impair autophagy or mitochondria remodeling, and does not affect adipogenesis *in vitro*. However, increased mitochondrial proteins and remodeling during 3T3-L1 adipogenesis are distinctly different from the adipogenesis *in vivo* or brown-to-white conversion in mouse adipose tissue. The above findings are summarized in Fig.3.5.1. Future studies to use primary adipocytes isolated from mice or human adipose tissues might be more suitable for study the effect of alcohol on adipocytes differentiation *in vitro*.

Figure 3.5.1

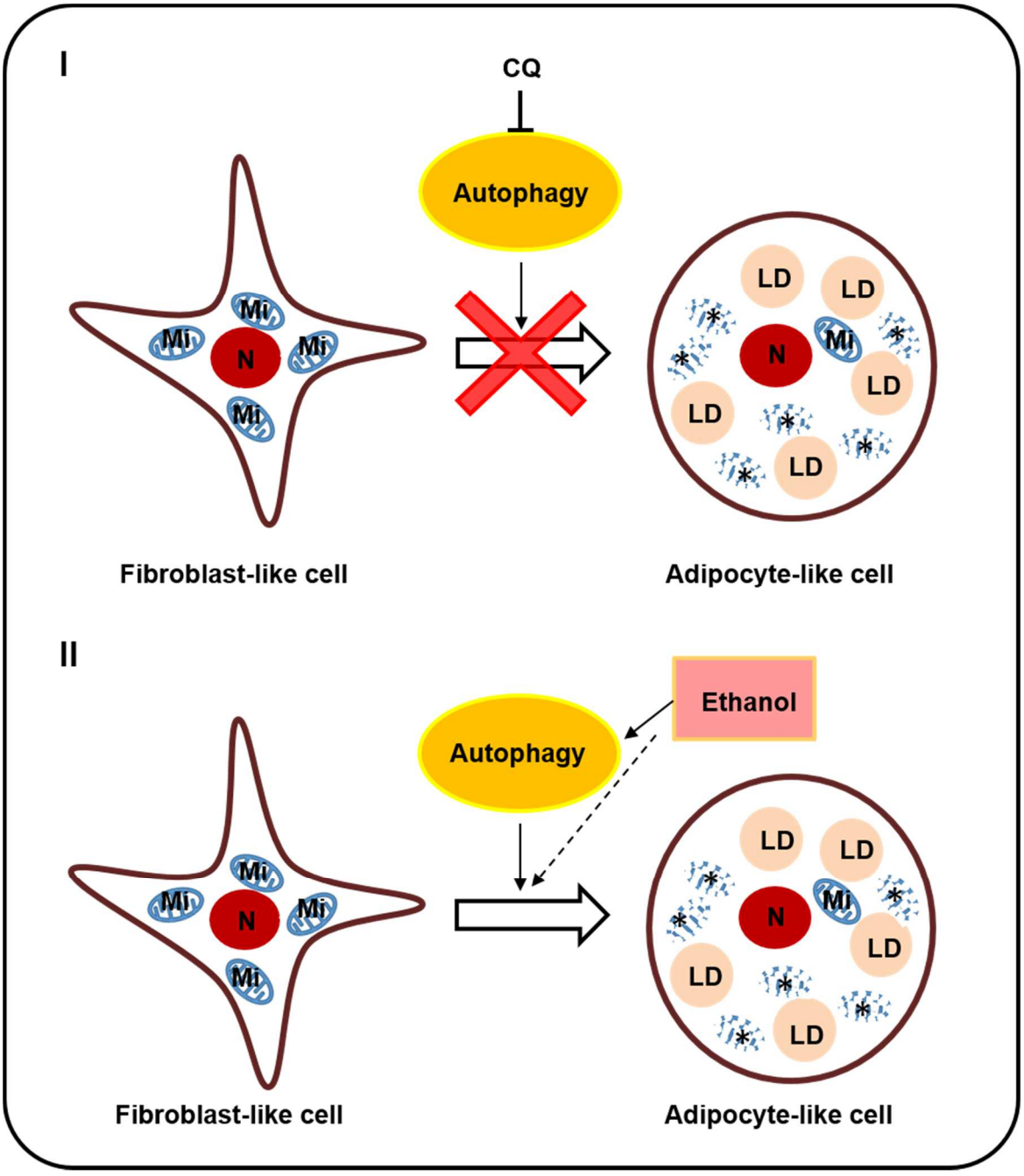


Figure 3.5.1 Scheme of adipogenesis in 3T3-L1 preadipocytes.

3T3-L1 preadipocytes change from fibroblast-like cells to adipocyte-like cells during adipogenesis, involving accumulated LDs, decreased typical mitochondria, and increased unidentified structures which might derived from mitochondria and/or lysosomes. I. The lysosome inhibitor CQ inhibits autophagy and blocks the remodeling of preadipocytes. II. Ethanol induces autophagy flux and does not impair the remodeling of preadipocytes. CQ: chloroquine; N: nucleus; Mi, mitochondria; LD, lipid droplet; *, mitochondria-like, lysosome-like structure.

CHAPTER 4. OVERALL DISCUSSION AND FUTURE DIRECTIONS

4.1 Concluding Remarks

In this study, we characterized the effect of chronic-plus-binge alcohol on mouse adipose-liver axis. In addition to the known induction of liver injury and steatosis, chronic-plus-binge alcohol exposure led to white and brown adipocyte atrophy, increased adipose lipolysis and FFA released into blood, and increased secretion of protective crosstalk mediators, adiponectin and FGF21. Mechanistically, we found that chronic-plus-binge alcohol inactivated Akt/mTOR signaling and increased autophagic degradation in eWAT. Adipocyte inflammation and cell death were not induced in eWAT and thus did not contribute to liver injury or steatosis in this alcohol model, unlike reported in chronic alcohol feeding model.

We investigated the role of adipocyte autophagy in adipose-liver axis in response to alcohol. Adipocyte autophagy inhibition by knocking out Atg5 did not significantly affect adipose mass. However, the KO mice were partially protected from alcohol-induced decrease in adipocyte size and elevation in liver ALT and AST, suggesting that adipocyte autophagy activation contributed to alcohol-induced adipose atrophy and liver injury. At the same time, the KO mice without alcohol exposure had increased basal levels of serum adiponectin and FGF21 compared to the WT mice, implicating that constitutional adipocyte autophagy inhibition led to altered adipokine and hepatokine secretion as an adaptive response. This supported the importance of autophagy in adipose function, and the significance of adipose-liver axis in ALD.

We next used the 3T3-L1 cells to study autophagy and adipogenesis *in vitro*. We found increased autophagy degradation during the differentiation process, and this process could be completely blocked by the lysosome inhibitor CQ, suggesting that the normal lysosome function was indispensable for adipogenesis. Interestingly, while in differentiated cells the typical mitochondria drastically decreased, there was increased mitochondria protein along with

appearance of many electron-dense vesicles. In addition, these cells had diffused mitochondrial staining with membrane potential. This indicates that during adipogenesis the mitochondria were not simply removed, and it is possible that the electron-dense vesicles were derived from mitochondria. If so, the differentiated 3T3-L1 cells were more like brown adipocytes rather than white adipocytes in terms of mitochondrial content. Therefore, 3T3-L1 cells might not well represent the whole picture of adipose tissues *in vivo*, considering white adipose tissues have much more depots and are more important in lipid mobilization and adipokine secretion than brown adipose tissue.

We also studied the effect of alcohol on 3T3-L1 cells. Ethanol or acetaldehyde of the dose we tested did not affect the induction rate of 3T3-L1 preadipocytes. This was consistent with the observation that ethanol or acetaldehyde did not impair autophagy flux or mitochondria remodeling, the two hallmark events in adipogenesis. In fact ethanol induced autophagy flux in differentiated 3T3-L1 cells, consistent with our observation *in vivo*. Besides, acetaldehyde did not affect autophagy flux or mitochondria protein during adipogenesis, suggesting that ethanol might pose effects on 3T3-L1 cells directly or via other metabolites.

4.2 Current Limitations and Solutions

4.2.1 Assessing Autophagy in Adipose Tissues in *In Vivo* Alcohol Models

In our study, we found decreased p62 and LC3-II proteins in eWAT after chronic-plus-binge alcohol. Since there was also inhibited Akt/mTOR signaling that suggests an overall catabolic status, we concluded chronic-plus-binge alcohol may increase autophagic flux in mouse adipose tissues. However, as mentioned in Chapter 1.3.1, autophagy is a highly dynamic process, and an autophagic flux assay using autophagy inhibitors is usually needed to accurately evaluate autophagy. Lysosome inhibitors (e.g. CQ, BAF, E64D+Pepstatin A) and lysosomal protease inhibitors (e.g. Leupeptin) that block autophagic degradation should be included in the chronic-plus-binge alcohol model in the future to further assess autophagic flux.

Another helpful model is to use the GFP-LC3 transgenic mice. In this model, autophagy marker LC3 is fused with GFP, so that autophagosomes characterized by GFP-positive puncta can be seen and quantified under fluorescent microscope in almost all the tissues except in the brain (Mizushima et al. 2004). Besides, during autophagic degradation free GFP proteins are cleaved from LC3-II, and free GFP fragments and GFP-LC3 proteins can be detected via western blot. Increased autophagic flux is usually associated with increased GFP-LC3 puncta, and increased free GFP fragments. Co-treatment using lysosome inhibitors could result in further accumulation of GFP-LC3 puncta. Using this model, our lab showed that protease inhibitor leupeptin further increased GFP-LC3 puncta, free GFP and GFP-LC3-II protein in mouse liver after chronic-plus-binge alcohol, indicating increased autophagic flux in liver by alcohol (Chao et al. 2018). Future studies will be helpful to also assess the changes of GFP-LC3 puncta and levels in adipose tissues after alcohol.

Since mCherry or mRFP signal is more stable than GFP against quenching and degradation, several reporter models have been developed including mRFP-GFP-LC3 transgenic mice (Kimura et al. 2007), αMyHC-mCherry-GFP transgenic mice (Iwai-Kanai et al. 2008), mCherry-GFP-LC3 transgenic mice (Terada et al. 2010), mice with mCherry-GFP-LC3 reporters delivered by AAV (Castillo et al. 2013), and GFP-LC3-RFP-LC3ΔG mice (Kaizuka et al. 2016). In these models, it is claimed that undegraded LC3 proteins have mCherry or RFP signal overlaid with GFP signal, and degraded LC3 proteins only have red signal. These models might be helpful to characterize autolysosome formation and degradation even without using lysosome inhibitors. It should be noted that mCherry or RFP signals still get degraded similar to GFP.

There are some concerns of this type of models in combination with lysosome inhibitors. Firstly, a saturated dose of lysosome inhibitors is needed for accurate interpretation of autophagic flux assay. This is because free GFP fragments are affected by not only autophagy stimuli, but also lysosome activity and lysosome pH (Ni et al. 2011). Free GFP fragments in GFP-LC3 stable cell line can increase even in the presence of lysosome inhibitors if the dose is not saturated. Similarly, mRFP-LC3 puncta in mRFP-GFP-LC3 transfected cells can increase with unsaturated dose of lysosome inhibitors. Secondly, lysosome inhibitors have to target the interested organs and induce measurable changes. Autophagic flux assay using GFP-LC3 tg mice and lysosome inhibitors has been proved practical in liver (Ni et al. 2012, Lim et al. 2014, Chao et al. 2018), heart (Iwai-Kanai et al. 2008, Terada et al. 2010) and pancreas (Chu et al. 2015) in alcoholic and non-alcoholic disease models, but the application in other tissues like adipose tissue lacks reports. In our hands, strong free GFP fragments were detected even without any treatment, and co-treatment with CQ or Leupeptin hardly increased LC3-II, p62, free GFP, or GFP-LC3-II proteins in eWAT and iBAT of GFP-LC3 mice (data not shown). This suggests that in fat there might be highly active

autophagic degradation and GFP-LC3-II cleavage, and CQ or Leupeptin might not be potent enough to block the degradation or cleavage, though the same dose of CQ or Leupetin works well in liver and pancreas. An appropriate dose of CQ or a more potent lysosome inhibitor targeting adipose tissue are needed for completing the autophagic flux assay in the future. Thirdly, currently we only assessed autophagy status in fat 8 h after binge, while autophagy activity might fluctuate over time during the feeding and after the treatment. Observations at different time points may lead to different conclusions.

4.2.2 *Ex Vivo* and Primary Cultured Cell Models for Studying on Alcohol and Adipose Tissue/Adipocytes

To better control the experiment conditions of study on alcohol and adipose tissue/adipocyte physiology, several *ex vivo* and *in vitro* models have been developed. eWAT explants isolated from mice after chronic alcohol feeding were found to release more FFAs during 2 hours' incubation in DMEM (Zhong et al. 2012). Mouse eWAT explants incubated with acetaldehyde for 2 h also released more FFAs, and explants from adipose-lipin1 KO were resistant to acetaldehyde-induced release of FFAs (Zhang et al. 2018). Besides, in rat eWAT explants incubated with acetaldehyde for 3 days, protein levels of lipogenic enzymes and regulators were decreased (Zhang et al. 2015). These observations prove that isolating eWAT explants from mice exposed to alcohol, and incubating eWAT explants with acetaldehyde, are practical ways to study the effect of alcohol on lipolysis and lipogenesis. In our study, we studied eWAT and sWAT *ex vivo*, and confirmed that the explants released more FFAs in response to isoproterenol-induced lipolysis, and the response was not significantly different in explants from A-Atg5 KO mice. It would be interesting

to see whether WAT explants from A-Atg5 KO mice are less sensitive to lipolysis after alcohol treatment in mice or acetaldehyde incubation with explants.

Ex vivo assay reflects adipose tissue physiology as a whole tissue, while different types of cells in adipose tissue can be isolated and studied respectively. Using collagenase, cells are digested from fat pad, centrifuged, are separated into floating mature adipocytes and pelleted SVF. SVF contains heterogeneous cell populations including preadipocytes [also called adipocyte progenitors or adipose-derived stem cells (ASCs)], immune cells (like macrophages and T cells), endothelial cells, circulating blood cells, pericytes, and fibroblasts. Two cell types from adipose tissue have been studied in the alcohol context: (1) Mature adipocytes: Mature adipocytes from rats with or without alcohol feeding have been used for glucose transport, adiponectin secretion and adipocyte lipolysis assays (Sebastian et al. 2008). Adipocytes from chronic alcohol-fed rats had no difference in basal lipolysis, less sensitive to β-adrenergic receptor-induced lipolysis due to decreased accumulation of cAMP, and less sensitive to insulin-induced inhibition of lipolysis (Kang and Nagy 2006, Kang et al. 2007). Adipocytes but not SVF have increased CYP2E1 protein after chronic alcohol feeding, which may contribute to apoptosis and inflammation in adipose tissue (Sebastian et al. 2011). (2) Preadipocytes: Preadipocytes are multipotent and can differentiate into adipocyte-like cells when incubated with adipogenic reagents. Preadipocytes isolated from chronic alcohol-fed rats showed impaired expansion and adipogenesis (Huff et al. 2011). In line with this, human adipocyte stromal cells had worse differentiation in the presence of ethanol (Crabb et al. 2011). It should be noted that the ratio and differentiation capacity of preadipocytes vary a lot due to fat depots, age, gene backgrounds, and environment (like diet) (Macotela et al. 2012). So far tno study has been reported to use preadipocytes from depots other than adipose tissue to study the impact of alcohol on adipocytes.

4.3 Future Directions

Firstly, we would like to further investigate the role of autophagy in adipose atrophy and alcohol-induced liver injury. As discussed in Chapter 2.5, we performed the constitutional *Atg5* deletion in adipose tissue. Adaptation in response to adipose autophagy deficiency are likely to have happened over 2 to 3 months in mice, and may mask the different response to alcohol in A-Atg5 KO mice. Acute autophagy knockdown via conditional KO models will help to focus on the consequences of adipose autophagy deficiency in ALD. As the effect of impaired differentiation in adipose tissue can be ruled out, a conditional KO model may be more relevant to ALD in humans.

Secondly, we are interested in expanding the crosstalk study to gut-adipose-liver axis in ALD. In this study we used epididymal WAT as a representative visceral fat. Mesenteric WAT, another depot of visceral fat, is attached to intestines in a web-like form, and embeds blood vessels and lymphatic vessels stemming from gut to portal circulation or systematic circulation. The importance of gut-adipose-liver axis has been proposed in metabolic syndromes like obesity and type 2 diabetes (Konrad and Wueest 2014). In obesity, fat-enriched diet alters gut microbiota (i.e. the community of microorganisms in gut) composition, impairs gut barrier function, and increases pro-inflammatory cytokine production (Winer et al. 2016) (Portune et al. 2017) (Hamilton et al. 2015). Increased bacterial particles and endotoxins may translocate into mesenteric WAT, stimulate adipocytes and resident immune cells to secrete pro-inflammatory cytokines, and predispose the body to obesity and insulin resistance (Cani et al. 2007). However, direct evidence is still lacking, and a few recent reports suggested that altered gut microbiota or gut barrier integrity was not important in diet-induced obesity and metabolic dysregulation in mice (Kless et al. 2015, Rabot et al. 2016). In ALD there is also altered dietary nutrient composition and microbiota, decreased gut permeability, and increased LPS translocation. It is intriguing whether there is

increased inflammation in mesenteric WAT, and if yes, whether it contributes to ALD like other adipose tissues. It should be noted that the findings related to gut microbiota in rodent models need to be translated to human cautiously, since microbiota composition varies a lot among species (Nguyen et al. 2015).

Thirdly, it will be interesting and important to study the effect of alcohol on adipose-derived microRNA (miRNA) and the therapeutic value of adipose miRNA in ALD. miRNA is a naturally occurring, small non-coding RNA encoded by nuclear DNA. It silences RNA and post-transcriptionally regulates gene expression. First described in 1993 (Lee et al. 1993), various miRNA populations have been found in different depots of adipose tissues (Kloting et al. 2009). miRNAs are associated with adipogenesis (Esau et al. 2004, Ortega et al. 2010), adipocyte fate determination (Sun et al. 2011, Mori et al. 2014), adipose inflammation (Ge et al. 2012, Ortega et al. 2015), lipid metabolism (Lin et al. 2014), obesity (Xie et al. 2009, Chartoumpekis et al. 2012, Ortega et al. 2013), and human immunodeficiency virus (HIV)-associated lipodystrophy (Mori et al. 2014, Squillace et al. 2014). miRNAs not only exert regulation in the cell *per se*, but also commute between different cells and distant tissues when they are excreted into exosomes, a type of cell-derived membrane vesicles (Valadi et al. 2007, Melo et al. 2014). Importantly, adipose tissue is a depot deriving circulating miRNAs which target multiple organs including liver. Recently Ying et al. found that miRNA-containing exosomes isolated from obese mice caused glucose intolerance and insulin resistance in lean mice, while exosomes from lean mice improved glucose intolerance and insulin resistance in obese recipients (Ying et al. 2017). Another recent study by Thomou et al. showed that mice with adipose-specific KO of miRNA-processing enzyme Dicer (*ADicer* KO), circulating miRNAs especially exosomal miRNA were extensively reduced, and fat transplantation reconstituted circulating miRNAs (Thomou et al.

2017). They further found that human with HIV-associated lipodystrophy also presented decreased circulating exosomal RNAs. Intriguingly, *ADicer* KO mice had increased tissue *Fgf21* mRNAs and circulating FGF21 levels, which could be reduced by BAT transplantation. As introduced in Chapter 1.4.4, FGF21 is a hormone mainly secreted in liver and may protect against liver injury and other metabolic dysfunctions. This study suggests that adipose-derived miRNAs are potential therapeutic targets of obesity and dysfunction in other tissues. Tissue miRNAs and circulating miRNAs are associated with alcohol-induced multi-organ injury including liver, pancreas, gut, brain, heart, and skeletal muscle (Natarajan et al. 2015). So far there is no report on the effect of alcohol on adipose miRNA or adipose-derived circulating miRNA. It remains to be studied whether any of these miRNAs would affect adipocyte autophagy, differentiation and lipolysis.

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